IDENTIFICATION OF THE KEY AROMA ACTIVE COMPOUNDS OF PROPOLIS COLLECTED FROM CENTRAL NEW JERSEY OVER THREE CONSECUTIVE YEARS

By

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ABSTRACT OF THE THESIS

Identification of the Key Aroma Active Compounds of

Propolis Collected from Central New Jersey

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Produced by bees, propolis has attracted substantial scientific interest and consumer demand as a "superfood". Long used in folk medicine, propolis is known for its purported health promoting properties and pleasant aroma. Propolis is consumed as a dietary supplement; it is also a raw material in chewing gums, confections, and honey, and a preservative added directly to food or integrated into packaging.

Although propolis volatiles have been studied for many years, little is yet known about the key aroma-active compounds responsible for its pleasant characteristic aroma. Research has shown that propolis volatiles vary with geographic location, time of collection, and plant source. However, no research has documented variability in the aroma active compounds of propolis from the same bee hive over consecutive years or investigated which compounds are key odorants with greatest influence on the unique aroma of propolis.

This research, therefore, aimed to identify key aroma active compounds in propolis and to gain insight into its seasonal variability. Aromas were isolated gently by solvent

assisted flavor evaporation (SAFE) and volatile components were separated by on-column gas chromatography mass spectrometry (GC-MS) in addition to purge and trap thermal desorption (P&T-TD-GC-MS). Although hundreds of volatiles were present and identified in the samples, gas chromatography olfactometry (GC-O) demonstrated that only 47 were aroma active. Application of comparative aroma extract dilution analysis (cAEDA) identified α-pinene (pine-like), 1,8-cineole (eucalyptus-like), (E)-2-nonenal (green), (E,Z)-2,6-nonadienal (cucumber-like), (E)-β-damascenone (cooked apple), 2-methoxyphenol (smoky), phenylethyl alcohol (floral, rose), β-ionone (floral, berry), eugenol (clove-like), (E)-ethyl cinnamate (cinnamon-like), 2-methoxy-4-vinylphenol (clove-like), and 3-phenylpropanoic acid (floral) as the most aroma-active compounds. Concentrations of individual components varied with season, but overall distribution of odorants was remarkably consistent over three years.

These results provide considerable insight into aroma compounds that contribute to propolis aroma and into seasonal changes in composition of those compounds. This knowledge should contribute significantly to applications of propolis to dietary, pharmaceutical, flavor and fragrance industries, and also set the stage for future research on the key aroma active compounds of propolis collected from different geographical locations as well as the development of aroma standardization and quality control methods.

Acknowledgements and Dedication

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This Research and Work is Dedicated to:

My husband, Krzysztof Tomaszewski. Thank you for constantly supporting me throughout this process. Thank you for helping my dreams to come true. Without your sacrifices and support, this thesis would not have been possible.

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1. Introduction

Propolis is a fragrant, sticky, and resinous plant-derived substance collected by bees as a caulking, sealing, lining, strengthening, and preserving material for hive construction [1]. Propolis is found inside the hive and around its entrance and may have a repelling or masking effect that protects bee colonies from certain pests and diseases. Propolis is collected by all species of *Apis* as well as stingless bees such as *Melipona, Trigona*, and so forth[2]. Foraging bees collect propolis substrates from the resinous exudates of woody trees and shrubs. It is thought that exudates of the genus *Populus* are preferred wherever they occur [3], although bees must collect material from a variety of plant species, depending on geography and seasonal availability. There is evidence of propolis collection from *Pinus spp.* [4] and desert composites [5]. Some studies have indicated that, although not a uniform substance, propolis composition is remarkably similar despite its differing origins [5, 6]. However, some propolis, such as Brazilian propolis, appears to have unique properties such as antitumor and antiviral activity. Moronic acid present in Brazilian propolis shows significant anti-HIV activity [7]. The Artepillin C, extracted from Brazilian propolis shows suppression on the growth of tumor cells [8]. The literature indicates the presence of compounds such as flavonoids in propolis with a broad spectrum of biological properties, including anti-inflammatory effects. A representative study has shown that dietary propolis suppresses the lipoxygenase pathway implicated in arachidonic acid metabolism during inflammation [9, 10].

The chemical composition of propolis, both its volatile and non-volatile constituents, varies from sample to sample. There are several known biologically active constituents of propolis, including flavonoids, phenolics, and aromatics [11]. Some

chemical constituents of note include cinnamyl alcohol, cinnamic acid, vanillin, benzyl alcohol, benzoic acid, caffeic acids, ferulic acids, phenolic triglycerides, pterostilbene, eugenol, caffeic acid phenolic ester, and caffeic acid pentenyl esters [2] (Figure 1).

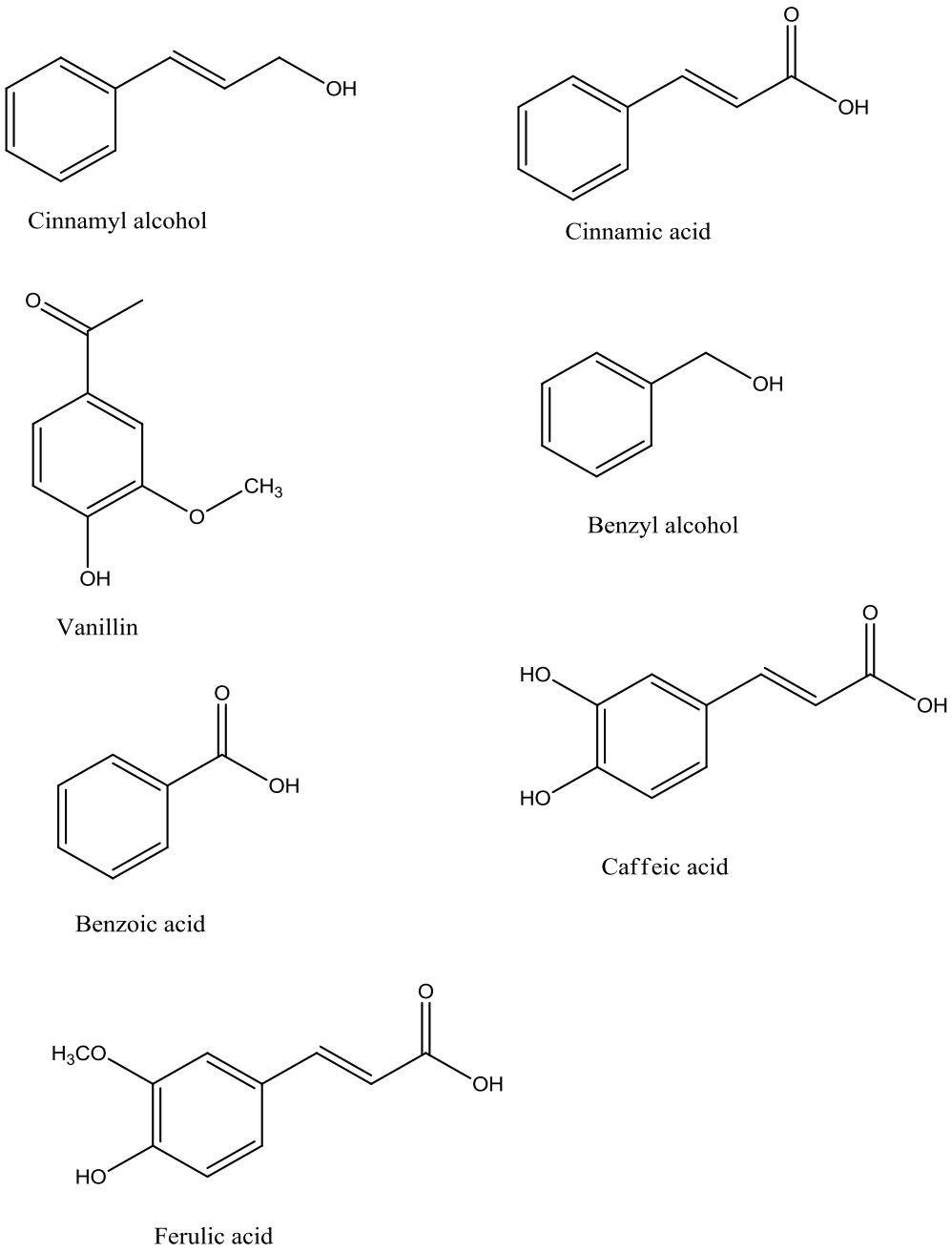

Figure 1. Examples of some of the bioactive constituents in propolis [2].

The antimicrobial properties of propolis are well documented [12] and the substance is being investigated for anti-cancer [13], immune activation [14], and other clinical uses. Propolis was used as a medicinal remedy in ancient Greece and has been discussed by the famous Italian philosopher and naturalist Pliny the Elder [15]. In recent years, preparations made from propolis have become increasingly popular in functional foods, dietary supplements, and cosmetics. Propolis is commonly ingested in the form of capsules, throat sprays, and tinctures, and can also be applied externally to the skin in the form of lotions and ointments [16].

In addition to its health-promoting properties, one important factor that contributes to the popularity of propolis is its pleasant aroma. Propolis has a highly fragrant scent that can be described as bee wax-like and honey-like with complex spicy, herbal, and floral nuances [17, 18]. Although a significant amount of scientific research has been conducted on the biological activities of both volatile and non-volatile constituents of propolis [19-21], very few studies have focused on the active compounds which contribute to its aroma. Moreover, to the best of our knowledge no research has been designed to determine the temporal variability of propolis in a single beehive over consecutive years.

For these reasons, the aims of this investigation are to identify the key aroma active compounds present in propolis and to gain insight into the seasonal variability of propolis odorants. To acomplishe this, propolis was sampled from a single beehive in central New Jersey over three consecutive seasons, from Spring 2011 through Spring 2013. Volatile fractions were isolated by solvent-assisted flavor evaporation (SAFE) distillation, aroma-active compounds were distinguished by gas chromatography olfactometry (GC-O), and

intensity and changes over time were determined by comparative aroma extract dilution analysis (cAEDA) of the volatile fractions.

2. Background

2.1. History of propolis

The word propolis comes from Greek origins and means “in the front of the city” [16, 22]. Propolis also represents "bee glue" in its role of cementing openings of a bee hive. It was used by ancient Egyptians, Persians, Romans, and Greeks, who depicted propolis-making bees on ornaments and used it to relieve a number of illnesses. Greeks used it as the principal ingredient in perfumes [23], and in ancient Egypt it was used as an adhesive. The Greek philosopher Aristotle referred to propolis as a substance for curing bruises and sores [23, 24].

The Roman scholar Pliny the Elder (23-79 A.D) knew about the use of propolis as a glue in bee hives and also about its medicinal properties. In his 35 volumes of *Natural History* [25], he stated, “Current physicians use propolis as a medicine because it extracts stings and all substances embedded in the flesh, reduces swelling, softens induration, soothes pain, and heals sores when it appears hopeless for them mend.”

In the Middle Ages, propolis was not popular and its use in medicine soon disappeared [22]. Only a few references to it in that time have survived. Some sources from the twelfth century described medicinal preparations containing bee glue for treatment of oral and pharyngeal infections as well as for dental cavities [26].

The interest in propolis returned to Europe during the Renaissance era. Thanks to medical humanists, some old and forgotten remedies and treatments were rediscovered and used again by healers like John Gerard and Nicholas Culpeper [16].

At the beginning of the 19th century propolis was studied and described by Nicolas Louis Vauquelin, a French pharmacist and chemist. The development of research on

propolis was connected with the development of chemistry. Examples include studies on the chemistry of flavonoids, a class of common polyphenols found in propolis [22].

Research on the chemical composition of propolis began in the 20th century. Early attempts to determine its composition were based on simple fractionation [22]. A number of German scientists developed methods for the extraction of different compounds, including vanillin, cinnamic acid, and cinnamyl alcohol. A series of studies conducted in the USA detected small amounts of vitamins B1, B2, B6, C, E, and nicotinic and pantothenic acid [22].

Studies on the chemical composition of propolis continued in the 1900s. At first, it was thought to be of a very complex chemical composition, but with a constant chemistry. However, the analysis of numerous samples from different geographic regions as well as the application of advanced laboratory methods showed that the chemical composition of propolis is highly variable. The composition of propolis is not fixed, and varies considerably from region to region, from season to season, and from hive to hive.

In late 20th century a series of medical studies with propolis were performed in Denmark, the results of which were found to be promising for treatment of number of illnesses [16]. For example, in the treatment of sore throat, propolis was effective and was well-tolerated with almost no side effects. After observing the therapeutic effects of propolis on more than 50,000 patients in Scandinavia, Dr K.Lund Aagard drew the following conclusions:

> The field of influence of Propolis is extremely broad. It includes cancer, infection of the urinary tract, swelling of the throat, gout, open wounds, sinus congestion, colds, influenza, bronchitis, gastritis, diseases of the ears, periodontal disease, intestinal infections, ulcers, eczema eruptions, pneumonia, arthritis, lung disease, stomach virus, headaches, Parkinson's disease, bile infections, sclerosis, circulation deficiencies, warts, conjunctivitis and hoarseness.[16]

It has been generally believed that bees produce propolis to protect the hive. Apart from their role in sealing, blocking holes and cracks, and smoothing out the internal walls, propolis also appears to act as an antiseptic in the prevention of microbial infection of larva, honey stores, and the combs. Because honeybee populations are very confined and the bees live in close contact, illness from one bee can spread quickly to the entire hive. Hives, yet stay healthy because the bees manufacture their own antibiotic, propolis, to reduce microbial growth on hive walls [16].

2.2 Collection of Propolis by Bees

Honey and other bees use their mouthparts to isolate sticky resin materials from tree leafs buds, shrubs, and other botanical sources as the starting material for synthesizing propolis. Bees collect the propolis substrates from a variety of plant species depending on seasonal availability [8]. They place collected resins into pollen baskets on their hind legs for transport to their hives, where other bees assist in removing the resinous material. The resins are then mixed with bee's wax and salivary enzymes to produce propolis which, as we now know, has many therapeutic properties [27, 28].

2.3 Physical Characteristics of Propolis

Propolis is a highly fragrant, soft, pliable, and sticky material collected by worker bees during warm and dry days. Propolis is also known as a "bee glue" because it is used by bees to seal cracks and empty spaces in their hives to protect them from intruders. It maintains the hive's waterproofing and helps regulate proper temperature and humidity inside the hive. Propolis is soft and malleable at moderate temperatures. However, when

cooled down to freezing temperatures it tends to become hard and brittle [7]. Its color may vary from green, black, yellow, red, and brown [9-14] (Figure 2) according to the trees, shrubs, and sap from which the resin was derived (Figure 2). The most commonly observed color of propolis collected from around the world are different hues of brown (from light to dark brown).

Figure 2. Example of different colors of propolis [29-32].

2.4 The Composition of Propolis

The chemical composition of propolis is very complex. The major components of propolis are resins, bee wax, essential oils, pollen, and other organics and minerals [33-35] (Table 1). The composition of volatile and non-volatile propolis constituents varies, based on the broad range of botanical sources visited by bees when collecting the resinous material. The most common plant source is poplar, followed by alder, birch, chestnut, ash, various *Prunus,* and willow [11, 19]. However, in some areas where these sources do not

exist, bees source the resins from other plants. At least 67 plant species have been identified as a source from which the honey bee has been reported to collect propolis [36]. Different bee species also may be a factor for its chemical diversity [37].

Table 1. The major components of propolis [38].

Class of Components	**Group of Components**	**Amount**
Resins	Flavonoids, phenolic acids and esters	45 – 55 %
Waxes and Fatty Acids	Beeswax and plant origin	25 – 35 %
Essential Oils	Volatiles	10 %
Pollen	Proteins (16 free amino acids > 1 %) arginine and proline together 46 % total	5 %
Other Organics and Minerals	14 trace minerals, iron and zinc most common ketones, lactones, quinones, steroids, benzoic acid, vitamins, sugars	5 %

The geographical location and the season of propolis harvesting may also influence its chemical composition [19, 39]. Over 300 compounds have been identified in different propolis samples and this number continues to increase with the growing number of propolis research applications [35, 38]. Huang et al., 2014, summarized some of the chemical categories reported in propolis from 2000 – 2012 (Table 2). Included are several bioactive natural products, including flavonoids, aromatic acids, phenolics, and terpenoids [16, 38, 40]. Structures of the main representative chemical components in propolis are shown in Figure 1.3 [11].

Table 2. The chemical categories reported in propolis since 2000 [11].

Chemical Category	Example Compound	Geographical Origin	Plant Source	Bee Species	References
Flavonoids	Luteolin	Australia, Brazil, Burma, Canada, Chinese, Cuba, Egypt, Greece, Japan, Kenya, Mexico, Nepal, Poland, Portugal, Solomon Island, Taiwan	*Populus*, *Macaranga*, *Dalbergia*	*Apis mellifera*	[41-54]
Prenylated flavanones	7-*O*-prenylpino-cembrin	Greece, Japan		*Apis mellifera*	[47, 49]
Neo-flavonoids	Cearoin	Nepal	*Dalbergia*	*Apis mellifera*	[55]
Monoterpenes Sesquiterpenes Diterpenes	Linalool abietic acid	Brazil, Greece, Indonesia, Iran, Malta, Turkey	*Ferula Pinaceae Cupressaceae*	*Apis mellifera*	[44, 56-60]
Triterpenes	Lupeol acetate	Burma, Brazil, Cuba, Egypt, Greece		*Apis mellifera*	[61-65]
Phenylpropanoids and esters	*p*-Methoxycinnamic acid	Australia, Brazil, Egypt, Uruguay	*Citrus*	*Apis mellifera*	[66-68]
Prenylated Phenylpropanoids	3-Prenyl-4-hydroxycinnamic acid	Brazilian green propolis	*Baccharies*	*Africanized Apis mellifera*	[69]
Stilbenes and prenylated stilbenes	3-Prenylresveratrol	Australia, Brazil, Greece, Indonesia, Kenya	*Macaranga*	*Apis mellifera*	[42, 44, 51, 66, 70]
Lignans	6-Methoxydiphyllin	Kenya		*Apis mellifera*	[44]
Coumarins	Prenylated coumarin suberosin	Iran		*Apis mellifera*	[58]

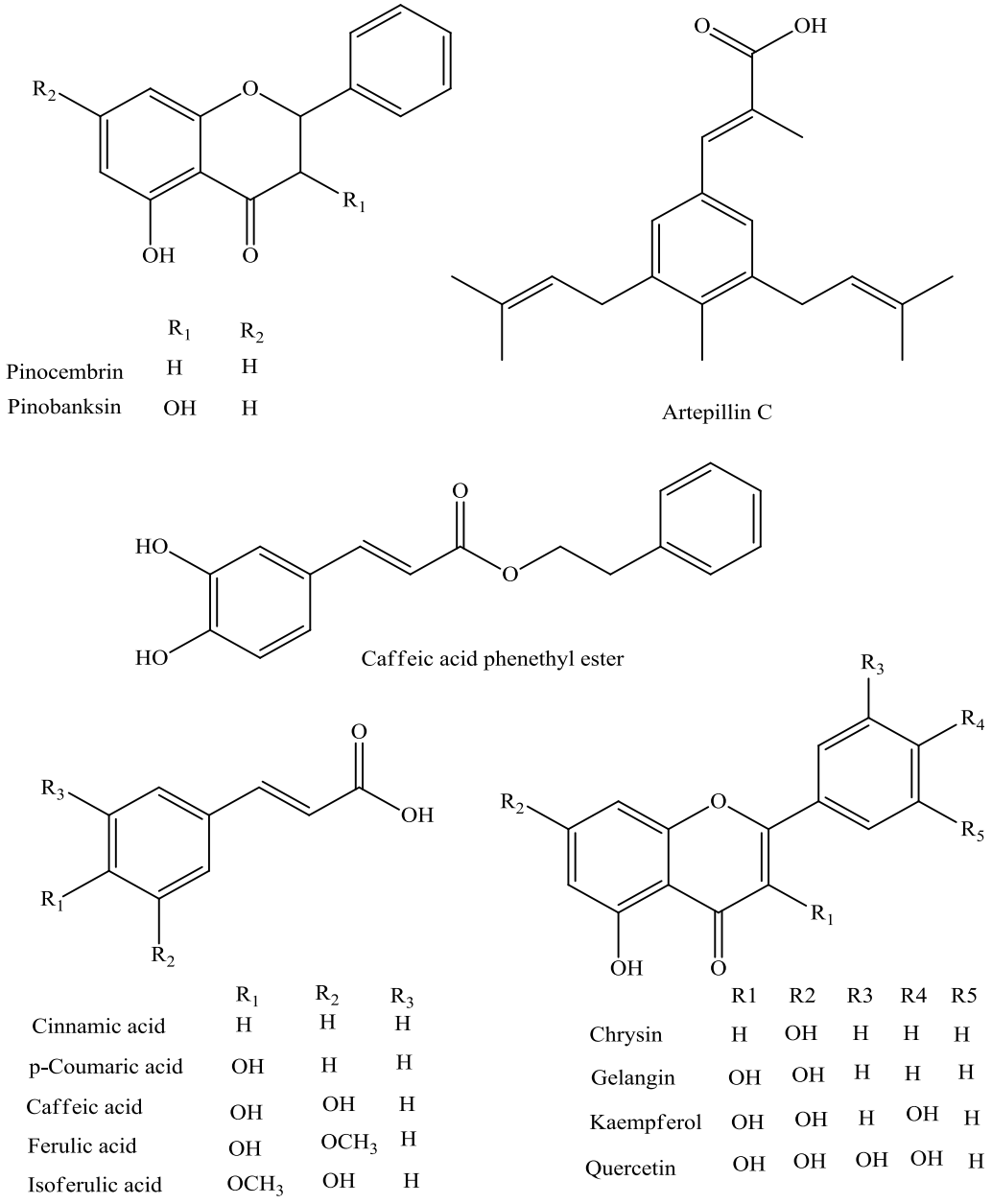

Figure 3. Structures of some compounds in propolis documented by Huang et al.[11].

2.4.1 Bioactive Properties of Propolis

Propolis has a long history of therapeutic use in traditional folk medicine. It continues to be commonly used as a remedy for rheumatism and muscular pain, skin problems, healing of wounds, asthma, and in dental care and general health maintenance [71]. It has been widely used as a folk remedy and recently was categorized as a superfood. This marketing term describes propolis as a food that may help certain medical conditions and improve human health. The widespread utility of propolis has attracted the cosmetic, pharmaceutical, and food industries. Also, scientific interest in propolis has expanded research significantly in recent years with additional studies demonstrating its antibacterial [72], antifungal [73], antiviral [11, 74], anti-inflammatory [75, 76], antioxidant [77-79], antitumor [34], and anti-HIV activities [7, 80]. Propolis types, chemical composition, and biological activities have been summarized by Bogdanov [23] and are presented in Table 3. Although the biological activities of compounds found in propolis are well documented, the effect that propolis on human health remains unknown, and remains an area for future research.

Table 3. Biological effect of propolis components.

Component	Propolis Type	Biological Activity	Reference
Polyphenols and flavonoids	Mostly poplar, but present in most propolis type	Antibacterial, antiviral, antifungal, antioxidant, antiaging, antiulcer, antitumor, antiallergic, anti-inflammatory, antiosterporotic, antitrombogenic, antiatherosclerosis, cardioprotective, immunomodulating, hepatoprotective, sicatrising	[39, 81-91]
Caffeic acid phenylethyl ester (CAPE) and other caffeates	Poplar, Baccharis	Antioxidant, anti-inflammatory, antitumor, antibacterial, antiviral, fungicide, immunomodulatory, cardioprotective, hepatoprotective, antiosteoporosis	[92-97]
Caffeic acid (CA)	Poplar, Baccharis	Antiviral, antioxidant, antiulcer, antitumor	[95]
Polyprenylated benzophenones	Cuba, Venezuela and Brazil	Antioxidant, anti-inflammatory, antitumor	[92, 93]
Artepillin C	Baccharis	Antioxidant, anti-inflammatory, antitumor, apoptosis inducing	[92, 93]
Prenylated flavanones (propolins)	Taiwan	Antioxidant, anticancer, apoptosis inducing	[92, 93]
Terpenes	Greece, Crete, Croatia, Brazil	Antibacterial, antifungal	[47, 98-101]
Essential oils	Brazil, Poland	Antibacterial	[72, 102, 103]
Furfuran lignans	Canary island	Antibacterial	[104]

2.5 Propolis Products and Their Use

2.5.1 Raw Propolis

Propolis may be purchased from local beekeepers and directly consumed in its raw form. At room or elevated temperatures, propolis has a soft, gluey consistency, and is therefore recommended to be consumed by chewing [38]. However, when cooled to freezing temperatures, propolis will harden and become brittle, which allows it to be ground into a fine powder (Figure 4). Propolis in this form may be added into a variety of food preparations such as salads, butters, and drinks.

Figure 4. Image of a ground propolis sample.

2.5.2 Liquid Extracts

Propolis in its liquid form is the most common commercial source. It may be consumed internally and externally. Propolis consumption is very popular worldwide, especially in Europe, and is found in many throat sprays, syrups, and tinctures (Figure 5). In those forms, usually a small amount of raw propolis is mixed with basic liquids such as alcohol, propylene glycol, water, or a variety of sweet extracts such as honey, glycerine, and maple syrup. The most commonly used solvent in propolis liquid extraction is aqueous ethanol.

Figure 5. Three examples of propolis liquid products [105-107].

2.6.3 Dietary Supplements

Due to its purported bioactive properties, propolis is consumed frequently as a dietary supplement. It may be found in soft and hard gel capsules (Figure 6). Most of the time, though, propolis as a dietary supplement is administered in powdered form, mixed with other bee hive products such as royal jelly, bee pollen, or other fillers. It may also be encapsulated in a two-piece hard gelatin [16].

Figure 6. Two examples of commercially available propolis as a dietary supplement [108, 109].

2.6.4 Propolis as an Additive

Propolis in its raw or extract form may be used as an additive in food, cosmetics, or medical industries. Ethanol extracts of propolis may be directly incorporated into other food preparations. In the food industry, propolis is incorporated as an additive into a chewing gums, lollipops, and honey [110].

Figure 7. Three examples of commercial propolis products [111-113].

2.6.5 Oral Health

Toothpastes, mouthwashes, breath fresheners, and chewing gums produced with the addition of propolis have been manufactured and used for oral hygiene (Figure 8). A small addition of propolis (~ 1%) in routine dental hygiene products such as toothpastes is incorporated for helping to treat dental cavities, gum inflammation, and dry socket [114-117].

Figure 8. Two examples of oral health products containing propolis [118, 119].

2.6.6 Food Technology

A major application area for propolis is in the food and beverage industry. A variety of patents cover the different application areas of its usage [120]. For example, in food technology propolis may be use as a preservative by direct addition into food or by its integration into food packaging [73, 121-126]. There are many documented applications of propolis as a food preservative [83]. Some of them indicate that propolis may extend the shelf life of frozen fish [127] and mashed potatoes [128]. A small addition of propolis (30 ppm) into a hen's diet may increase her egg production. The study also shows that by adding a small amount of propolis (500 ppm) in the diet of broiler chickens increased weight gains by up to 20% [129]. Propolis also has applications for other food groups such as wines and spirits [130] (Figure 9), breads, and butter or vinegar.

Figure 9. Two examples of alcoholic beverages containing propolis [131, 132].

2.6.7 Cosmetics

The cosmetic industry is another big market for a propolis applications. Propolis is used in creams and ointments for external afflictions such as skin problems, eczema, wounds, burns [133], as well as in anti-aging creams [38]. It may also be found in applications to soaps and shampoos (Figure 10). Blending of propolis into basic soaps or shampoos may improve skin and hair conditioning, although more research is needed to verify efficacy in this application.

Figure 10. Three examples of cosmetic products of propolis [134-136].

3 Techniques Used for Aroma Analysis

The combination of aroma, taste, and trigeminal sensation influence our perception of food flavor. Since aroma is the major aspect of the food flavor, it is important to determine both compounds contributing to aroma and the characteristics each imparts. Food flavor is very complex and no single technique provides all essential information, because each method has limitations as well as biases. Thus, multiple approaches must be integrated to develop a more complete profile of propolis aroma, their flavor/aroma characteristics, and their relative impact.

3.1 Sensory Techniques

Although sensory testing is subjective, it is critical for determining how humans perceive the characteristics of food flavor. In this method a food or specific flavor compound is tasted and evaluated based on human perception rather than instrumental analyses.

3.2 Analytical Techniques

3.2.1 Aroma Isolation

In order to analyze samples by gas chromatography (GC) the aroma sample must be innately volatile or be converted into a gas phase via heating. Selection of the appropriate aroma isolation technique, the selection and preparation of the sample matrix, sample concentration, formulation complexity, and variation of volatility in addition to the stability of the aroma compounds at high temperatures are all critical factors that must be considered prior to GC analyses of aroma compounds. Although a wide range of isolation

techniques may be applied for aroma isolation, in recent years solvent extraction, solvent assisted flavor evaporation (SAFE) and purge and trap/thermal desorption (P&T-TD) are common techniques used for isolation of aroma compounds prior GC analyses [137].

3.2.1.1 Solvent Extraction

Solvent extraction is a simple and efficient approach for aroma isolation [138]. A food sample is mixed with appropriate organic solvent and agitated to dissolve volatile constituents. Since the solvent extraction step will also isolate lipids and other organics from the food sample, the SAFE technique is one approach to isolate the volatile aroma compounds from the non- volatile organic components extracted from the food sample. Diethyl ether is a good, universal organic solvent that readily extracts organic esters, aldehydes, ketones, and alcohols from their natural sources [137]. However diethyl ether can readily form explosive peroxides and is flammable. Stabilizers such as 2,6-Di-*tert*-butyl-4-methylphenol (BHT) are added to increase the shelf-life of ether, but extreme care and safety still must be practiced during distillation. Moreover, diethyl ether is poorly miscible in water. This makes diethyl ether very useful in liquid-liquid extractions, but a drying step with magnesium or sodium sulfate is also required to remove water impurities and facilitates vacuum concentration of samples. The concentrated, crude aroma extracts are then analyzed by gas chromatography either directly or after separation and concentration of volatile compounds in the extract.

3.2.1.2 Solvent Assisted Flavor Evaporation (SAFE)

One method for isolating volatile aroma compounds from non-volatiles in solvent extract or complex food matrices is solvent assisted flavor evaporation (SAFE) [139]. In this method, the SAFE apparatus is connected to a high vacuum pump that allows for effective aroma isolation from various food matrices, even those with high oil content. This gentle technique also does not produce or has a minimal production of thermally induced artifacts.

When extracting volatiles through solvent assisted flavor evaporation (SAFE), the advantages and potential biases of the technique need to be taken into consideration. SAFE is a relatively easy technique to apply with a generally high recovery of odorants. In addition, this system is operated under reduced pressure at lower temperatures, which reduces the opportunity for artifact formation. However, this technique requires drying of the aroma isolate over anhydrous sodium sulfate to remove any water that may still be present, as well as concentration of the aroma isolate on a *Vigreux* column where the high volatile compounds may be lost. Moreover, the solvent peak possibly overlays peaks of isolated high volatile compounds which present themselves at the beginning of GC-MS spectrum. Due to these potential biases, the overall volatile profile of the sample may not express the complete picture

3.2.1.3 Purge and Trap Thermal Desorption (P&T-TD)

Purge and trap thermal desorption is a dynamic headspace concentration technique. A tube filled with absorbent material traps and concentrates the head space vapor volatile compounds (Figure 11). This technique is fast and is easily applied without the use of

solvents, which eliminates the solvent residual peak [137]. However, the high temperature applied in the purging and thermal desorption step may produce artifacts. In addition to artifact formation the purging efficiency and carry over may also pose an issue. The advantages of this technique are as follows: minimal sample preparation, elimination of the solvent peak, ability of trapping both high and low boiling molecules, and also relatively short time of sample analysis.

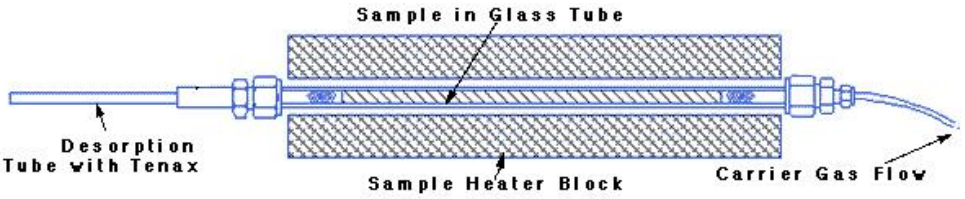

Figure 11. Purge and trap vessel used for the isolation and concentration of the volatile and semi-volatile compounds from solid samples. (Illustration courtesy of Scientific Instruments Services, Inc., Ringoes, NJ).

Tenax (poly[2,6-diphenyl-*p*-phenylene oxide]) is the most common material used in the purge and trap technique. Stable at high temperatures without any noticeable decomposition, it is a porous polymer with a very large surface area. It has high absorptive capacity of volatile and semi volatile organic compounds and low affinity for absorbing water. Other common sorbents used in purge and trap technique are Glass Beads, Carbosieve, Carboxen, and Carbotrap. Selection of the materials used for trapping of the volatile compounds depends on the sample and compounds of interest. While, Glass Beads are more useful for trapping the large molecular weight compounds, Carboxen is ideal for

trapping the smaller organic compounds. Tenax, in comparison to Carboxen and Glass Beads, may be more useful for trapping the volatiles from high moisture content samples.

3.2.2 Aroma Analysis

3.2.2.1 Gas Chromatography-Olfactometry (GC-O) for Identification of Odor-active Compounds and Characterization of Aromas.

Volatile isolates are extremely complex, containing hundreds of different volatiles, not all of which are aroma-active. Gas chromatography-olfactometry (GC-O), used to determine which components are aroma active and to assign specific descriptors to the aromas. It was the most widely used technique in this study. GC-O works similarly to traditional GC, except the capillary column is split by a Y-connector that directs part of the column effluent to the flame ionization detector and part to the sniffing port (Figure 12). The detector at the sniffing port is the human nose that both identifies when an eluting compound has a smell and assigns descriptors to the aroma. Many volatile aroma-active compounds have a very low threshold, i.e. they can be smelled at very low concentrations. These may be poorly detected by GC but have strong responses from the human nose.

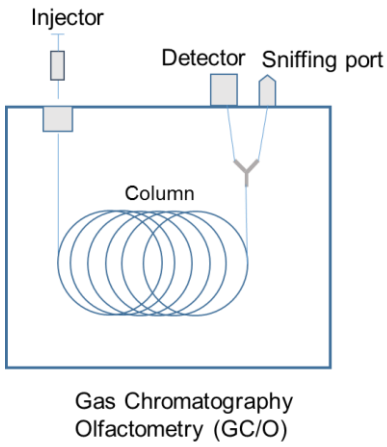

Figure 12. Gas Chromatography - Olfactometry (GC-O) diagram.

3.2.2.2 Aroma Extract Dilution Analysis (AEDA)

GC-O identifies compounds with aroma but does not determine relative importance or intensity of the individual aroma components. Aroma extract dilution analysis (AEDA) provides the quantitation. The sample extract is diluted 1:1 with solvent (v/v) sequentially to generate a series in which the dilutions are 2^n (Figure 13). Each dilution is then analyzed by GC-O in sequence of descending concentration until no additional aroma is detected by GC-O [140]. Each of the compounds with the highest flavor dilution factor (FD) detected by GC-O, typically 256 to 1024, is considered as a key odorant.

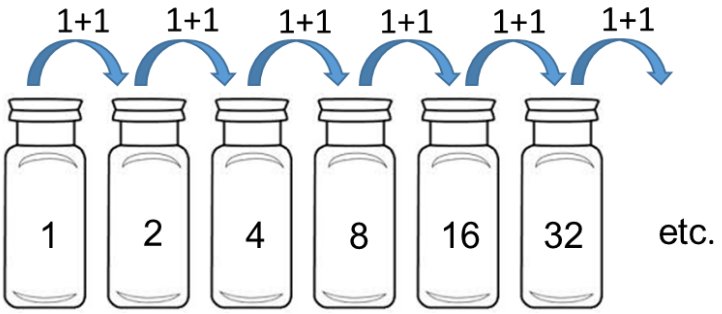

Figure 13. Stepwise dilution of the solvent extract with solvent (1:1. v/v) representing factor dilutions (FD) in 2^n increasing order.

3.2.2.3 Aroma Isolate Fractionation

The fractionation of the aroma isolates separates the complex mixture of the compounds into classes of chemicals with similar physical and chemical properties.

3.2.2.3.1 Solid Phase Extraction (SPE)

Selection and activation of the appropriate SPE material are critical for maximizing separation of analyte compounds. Giga tube packed with Si-1 silica material provides a strong polar compound selectivity. The dry packed SPE material is activated by organic solvents, including pentane, diethyl ether, or other solvents to condition the sorbent surface for interactions with specific types of compounds. The sample is loaded onto the column for concentration, then individual fractions are eluted by washing the column with a series of solvents of increasing polarity. SPE can be very useful for simplifying later analyses by separating complex mixtures into several simpler fractions of similar properties.

3.3 Identification of Molecular Structures for Volatile and Semi-volatile Aroma Compounds

Gas Chromatography-Mass Spectrometry (GC-MS) is a widely used analytical technique for the separation and identification of volatile compounds according to their mass-to-charge ratios. The effective combination of these two analytical techniques provides a high resolving power for the separation and identification of the aroma components of complex volatile mixtures. This powerful technique requires a small sample amount and gives a quantitative trace analysis (ppm, ppb) for the identification of compounds with a wide molecular weight range.

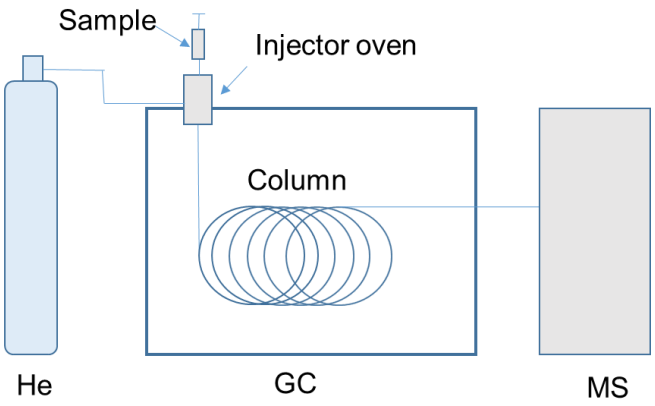

Figure 14. Gas Chromatography - Mass Spectrometry (GC-MS) diagram.

3.3.1 Gas Chromatography (GC)

Gas Chromatography separates volatiles according to their vapor pressures and their interactions with the carrier gas and the stationary phase. Gas chromatography has a very high resolution power. It can separate compounds that have 0.1 °C difference in their boiling points, or that have the same boiling point but different structures, i.e., isomers. It is a highly accurate and highly reproducible technique. The carrier gas which is used as a mobile phase must be of exceptionally high purity, and must be chemically inert, particle free, and suitable for detector analyses. The most common carrier gas is helium. It is not flammable, is ultra-pure, and applicable with most detectors. Helium has a small atomic diameter which helps the gas flow through a capillary column without producing much friction or resistance. The capillary column forms an integral part of the GC, where compound separation is achieved. The stationary phase inside the column separates the mixture of the volatile compounds into its individual components. There are a variety of columns and stationary phases that may be use for aroma analysis. As the separated compounds leave the column in the flow of the carrier gas they are analyzed by the detector.

The selection of the column and detectors used in the aroma analysis depends on the analyzed sample and the goal of the analysis.

3.3.1.1 Gas Chromatography Columns

The Free Fatty Acid Column (FFAP) is a high polarity column known for its excellent thermal and chemical stability. This column has a high resolution for free fatty acids and is one of the most popular column choices in GC food analysis. The peak sharpness for simple acids, organic acids, free fatty acids, and alcohols is improved when FFAP column is used.

ZB-5 is a low polarity column used in a wide range of separations such as essential oils, flavors, phenols, pesticides, etc. It is known for a long column life and is recommended for dirty or unknown samples.

3.3.2 Mass Spectrometry for Identification of Volatile Compound Structures

Mass spectrometry is an analytical tool used for compound identification. The ions of organic and inorganic compounds are separated by their mass-to-charge ratio (m/z), which allows for qualitative and quantitative detection by their m/z and abundance, respectively [141]. Mass spectrometry can provide information about sample molecular weight in its ionized form, and about structural information based on fragmentation patterns detected in the mass spectra or from GS-MS techniques.

4 Previous Research on Aroma-active Compounds in Propolis

A significant amount of research has been done on propolis, illustrating the vast interest this unique natural bee product has generated in the scientific world. Even though volatile and non-volatile constituents of propolis have been identified, there is still not much known about compounds that are aroma active. Headspace and GC-MS analyses have shown that a single propolis sample may contain over 150 volatiles [142]. From a wide variety of studies conducted on the volatile compounds, only a single study was found on the propolis aroma active compounds [17]. In this study, propolis from 23 regions of China had 44 identified compounds (Table 4) responsible for the unique propolis aroma in that region. However, these results may not be fairly compared with propolis samples from different geographic locations.

Table 4. Aroma active compounds identified in propolis from 23 different regions of China. Data from Yang et al., 2010 [17].

no.	aroma compound	RI	odor quality
1	ethyl acetate	889	fruity, sweet
2	pentanal	990	pungent, almond-like
3	α-pinene	1033	fresh, terpeney
4	toluene	1046	rubber, pungent
5	2-methyl-3-buten-2-ol	1051	fruity, faint scent
6	hexanal	1054	pungent, green, grassy
7	camphene	1055	aromatic, camphoric
8	butyl acetate	1085	fruity
9	ethyl butanoate	1094	apple, fruity
10	3-carene	1144	resin
11	β-myrcene	1157	ethereal, soapy
12	D-Limonene	1197	orange peel-like, fresh
13	β-pellandrene	1209	terpeney
14	eucalyptol	1212	camphoric
15	ethyl hexanoate	1236	apple peel, fruit
16	3-methyl-1-butanol	1239	malt, caramel
17	styrene	1246	rubber, bitter
18	3-methyl-3-buten-1-ol	1260	plastic, pungent
19	octanal	1290	soapy, fruity
20	2-methyl-2-buten-1-ol	1324	malty, bitter
21	1-pentan-3-ol	1335	pungent
22	1-hexanol	1351	grassy, floral
23	dipropyl disulfide	1366	pickled, meat
24	nonanal	1383	green, slightly pungent
25	acetic acid	1421	acidic
26	1-octen-3-ol	1449	mushroom
27	furfural	1476	pickled, roasted
28	α-cubebene	1482	herb, wax
29	propanoic acid	1503	acidic
30	benzaldehyde	1510	oily, almond
31	dihydro-5-methyl-2(3H)-furanone	1607	popcorn, caramel
32	butanoic acid	1616	sweaty, rancid, yoghurt
33	butyrolactone	1631	acetone, rancid
34	1,2,3,5,6,8a-Hexahydro-4,7-dimethyl-naphthalene	1647	grassy, pungent
35	naphthalene	1723	tar, camphoric, greasy
36	2-hydroxy-benzoic acid formate	1741	herb, mint
37	2-phenylethyl acetate	1767	oily, stale
38	2-methyl-2-butenoic acid	1777	greasy, acidic

Table 4. Continued. Aroma active compounds identified in propolis from 23 different regions of China. Data from Yang et al., 2010 [17].

no.	aroma compound	RI	odor quality
39	1-methyl-naphthalene	1784	pungent, rancid
40	benzyl alcohol	1894	burnt, faint floral
41	phenylethyl alcohol	1920	rose, honey
42	phenol	1968	phenolic
43	guaiol	2022	smoky, bitter, woody
44	cedrol	2046	camphoric, cool

5 Specific Objective and Significance of This Study

5.1 Specific Aims

The main objective of this study was to identify the aroma active compounds of propolis collected in central New Jersey and to determine the variability of composition in a single hive over three years (Springs of 2011, 2012, and 2013). Subtasks to accomplish this include the following:

1. Isolate volatile compounds from propolis by solvent assisted flavor evaporation (SAFE) and purge and trap - thermal desorption (P&T-TD)
2. Use Gas Chromatography-Olfactometry to determine which volatile components are aroma active and to assign aroma descriptors to each.
3. Use comparative aroma extract dilution analysis (cAEDA) to determine which odorants in propolis elicit the strongest aroma response (over 3 year period).
4. Characterize human aroma perception by free profiling and quantitative descriptive sensory analyses.

5.2 Significance

One of the important factors that is especially characteristic of propolis is its unique pleasant resinous aroma. This exceptional property contributes to the increasing popularity of propolis use. The composition of volatiles present in propolis is very complex, as is reflected in the many (>100) compounds that have been identified so far [142]. Research has shown that the bioactive components of propolis vary with geographical location, season, and botanical sources. However, additional research is needed to identify its aroma active constituents. Since one of the major application areas of propolis is the food industry,

it is significantly important to study and understand the complex composition of odor active compounds present in propolis both to understand what compounds are being added to food in propolis and to provide a basis for future standardization and quality control in use of propolis.

6 Experimental Procedures

6.1 Overview of the Study

Experimental design of this study contains two parts: sensory and analytical testing. Sensory testing such as Free Choice Profiling and Quantitative Descriptive Analysis (QDA) were based on the human perception and were not part of instrumental analysis. The second part of experimental design contains analytical testing where instrumental analysis were applied. Figure 15 shows the work flow diagram of this study approach. The actual sequence in which the analytical testing were developed was 1) isolation of the volatile compounds by solvent assisted flavor evaporation (SAFE) and purge and trap thermal desorption (P&T-TD); 2) fractionation of the aroma isolate by solid phase extraction (SPE); 3) analyzes of volatile isolates by gas chromatography mass spectrometry (GC-MS) on FFAP and ZB-5 capillary columns; 4) identification of the aroma active compounds by gas chromatography olfactometry (GC-O); 5) identification of the aroma key odorants by comperative aroma extract dilution analysis (cAEDA).

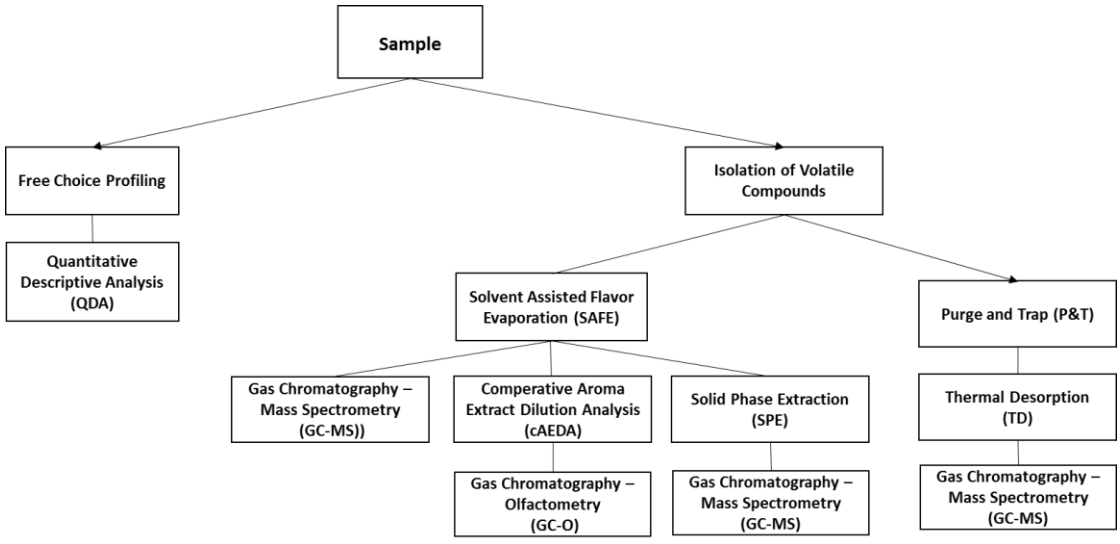

Figure 15. Experimental work flow diagram.

6.2 Sample of Propolis

The propolis samples analyzed in this study were collected from a suburban area located in central New Jersey. The samples were collected from the same bee hive (Figure 16) located at Wolgast Tree Farm and Apiary located at Somerset, New Jersey.

Figure 16. Bee hive located at Wolgast Tree Farm and Apiary, Somerset, NJ.

Propolis samples were collected from the same location, in the same season, over three consecutive years. The first sample was collected during the Spring of 2011, the second during the Spring of 2012, and the third during the Spring of 2013.

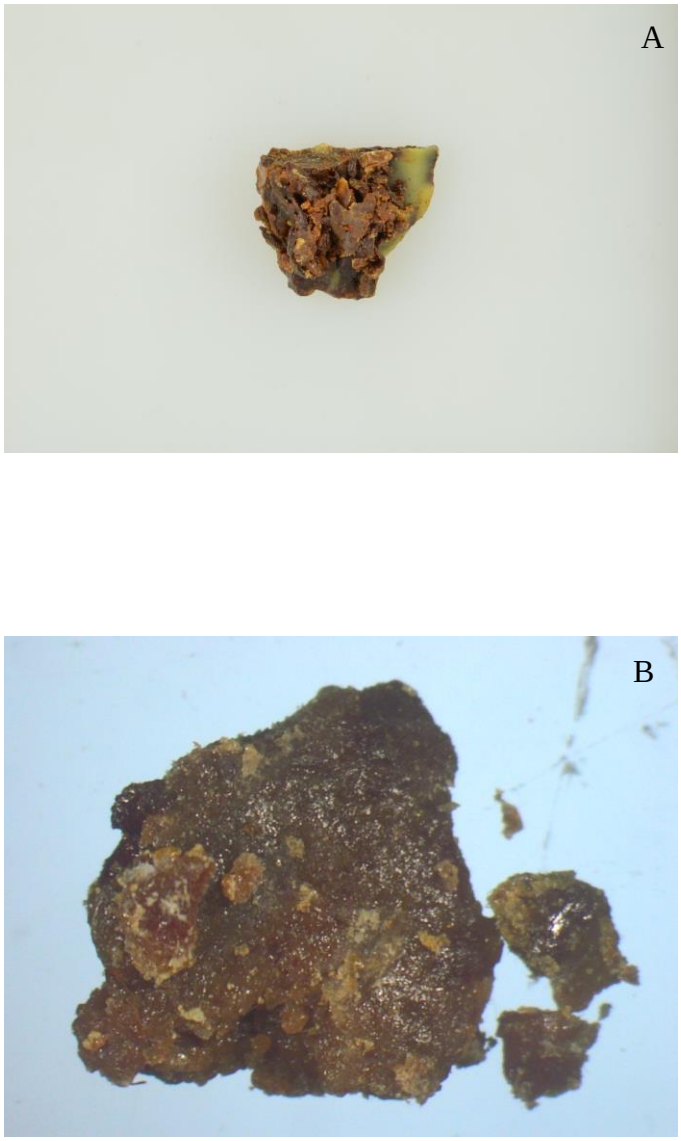

Figure 17. A) Image of propolis sample (Year 1) collected during the Spring of 2011. B) Propolis Year 1, magnification 15x.

Figure 18. A) Image of propolis sample (Year 2) collected during the Spring of 2012. B) Propolis Year 2, magnification 15x.

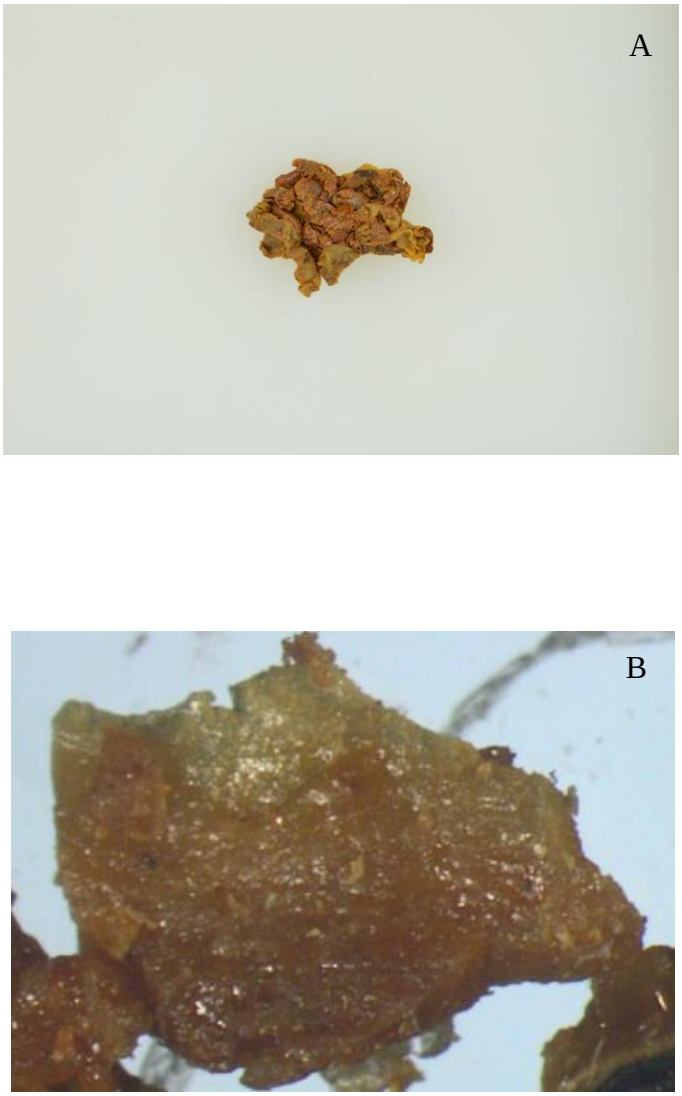

Figure 19. A) Image of propolis sample (Year 3) collected during the Spring of 2013. B) Propolis Year 3, magnification 15x.

Samples were collected by removing the propolis from the hive's honeycomb frames with a hive tool then were placed in glass jars and stored in the refrigerator (~4°C) until analysis.

6.3 Chemicals and Reference Aroma Compounds

Chromatographic grade diethyl ether was obtained from Honeywell Burdick & Jackson (Muskegon, Ml) and was freshly distilled in-house prior to use. Pentane was obtained from Fisher Scientific (Pittsburgh, PA) and also was freshly distilled in-house prior to use. Anhydrous Granular Sodium Sulfate was also obtained from Fisher Scientific. Helium Ultra High Purity and Liquid Nitrogen were obtained from Airgas.

The following reference standards (Table 5) were obtained from commercial suppliers 1 – 18, 20 – 26, 29 – 37, 39 – 42, 44 – 47 (Sigma Aldrich, St. Louis, MO); 19 &43 (Penta Manufacturing Company, Livingston, NJ); 27 & 38 (Vigon International, East Stroudsburg, PA). The trans-4,5-epoxy-(E)-2-decenal [143] was synthesized as described in the literature.

Table 5. List of the reference aroma compounds used for the compounds identification

No.	Name	CAS #	Supplier
1	hexanal	66-25-1	Sigma Aldrich
2	(E)-3-hexenal	6789-80-6	Sigma Aldrich
3	α-pinene	80-56-8	Sigma Aldrich
4	γ-terpinene	99-85-4	Sigma Aldrich
5	1,8-cineole	470-82-6	Sigma Aldrich
6	3-methylbutanal	590-86-3	Sigma Aldrich
7	1-octen-3-one	4312-99-6	Sigma Aldrich
8	dimethyl trisulfide	3658-80-8	Sigma Aldrich
9	acetic acid	64-19-7	Sigma Aldrich
10	2,3-diethyl-5-methyl pyrazine	18138-04-0	Sigma Aldrich
11	(E)-2-nonenal	18829-56-6	Sigma Aldrich
12	linalool	78-70-6	Sigma Aldrich
13	2-methylpropanoic acid	79-31-2	Sigma Aldrich
14	(E,Z)-2,6-nonadienal	557-48-2	Sigma Aldrich
15	butanoic acid	107-92-6	Sigma Aldrich
16	phenylacetaldehyde	122-78-1	Sigma Aldrich
17	2- and 3-methylbutanoic acid	503-74-2	Sigma Aldrich
18	(E,E)-2,4-nonadienal	5910-87-2	Sigma Aldrich
19	3-methyl-2,4-nonadione	815-57-6	Penta
20	phenylethyl acetate	103-45-7	Sigma Aldrich
21	(E)-β-damascenone	23726-93-4	Sigma Aldrich
22	hexanoic acid	142-62-1	Sigma Aldrich
23	2-methoxyphenol (guaiacol)	90-05-1	Sigma Aldrich
24	ethyl-3-phenylpropanoate	2021-28-5	Sigma Aldrich
25	phenylethyl alcohol	60-12-8	Sigma Aldrich
26	β-ionone	14901-07-6	Sigma Aldrich
27	δ-octalactone	698-76-0	Vigon International
28	trans-4,5-epoxy-(E)-2-decenal	134454-31-2	Synthesized
29	γ-nonalactone	104-61-0	Sigma Aldrich
30	4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF)	3658-77-3	Sigma Aldrich
31	cinnamaldehyde	104-55-2	Sigma Aldrich
32	4-methoxybenzaldehyde	123-11-5	Sigma Aldrich
33	methyl cinnamate	103-26-4	Sigma Aldrich
34	cinnamyl formate	104-65-4	Sigma Aldrich
35	4-methyphenol	106-44-5	Sigma Aldrich
36	γ-decalactone	706-14-9	Sigma Aldrich
37	(E)-ethylcinnamate	103-36-6	Sigma Aldrich
38	cinnamyl acetate	103-54-8	Vigon International
39	eugenol	97-53-0	Sigma Aldrich
40	4-ethyl phenol	123-07-9	Sigma Aldrich
41	2-methoxy-4-vinylphenol	7786-61-0	Sigma Aldrich
42	2,6-dimethoxy phenol	91-10-1	Sigma Aldrich
43	cinnamyl alcohol	104-54-1	Penta
44	trans-isoeugenol	97-54-1	Sigma Aldrich
45	phenylacetic acid	103-82-2	Sigma Aldrich
46	4-hydroxy-3-methoxybenzaldehyde (vanillin)	121-33-5	Sigma Aldrich
47	3-phenylpropanoic acid	501-52-0	Sigma Aldrich

6.4 Experimental Methods

6.4.1 Free Choice Profiling

Free choice profiling was performed first to determine a sensory lexicon for describing propolis samples. The sensory evaluation was carried out by an expert panel that performs sensory evaluation on a daily basis. Propolis samples (1 g) were crushed into small pieces and placed in 20 mL glass scintillation vials (Thermo Fisher Scientific, Fair Lawn, NJ). The seven panelists were asked to smell each propolis sample (one sample at a time) and describe the odor of the given sample. This test was repeated on samples from each year (propolis year 1, propolis year 2, and propolis year 3). The sensory characteristic descriptors were combined and the most common descriptors were then used as the basis for quantitative descriptive analysis.

6.4.2 Quantitative Descriptive Analysis (QDA)

Quantitative descriptive analysis (QDA) test was performed by seven trained panelists. Propolis samples (1 g) were placed in glass scintillation vials and provided to the panelists for orthonasal evaluation. All the samples (propolis year 1, year 2, year 3, and reference compounds dissolved in water) were given to the sensory panelists at the same time. The nine reference compounds, selected from free choice profile results, were (Z)-3-hexenal (green), butyric acid (cheesy, sweaty), phenylacetaldehyde (floral), ethyl cinnamate (cinnamon), 1,8-cineole (eucalyptus), eugenol (clove), α-pinene (piney), 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) (caramel), and phenyl acetic acid (honey).

6.5 Isolation of Volatile Compounds by Solvent Assisted Flavor Evaporation (SAFE)

Solvent assisted flavor evaporation (SAFE) technique was used for gentle aroma isolation. This technique allows for the separation of the volatile compounds from the non-volatile components of the sample while producing minimal artifacts. The aroma isolation unit contained three parts (Figure 20): the vacuum system, heating system and SAFE glass unit. The SAFE apparatus was connected to a high power vacuum, Leybold BMH Turbo System controller (Leybold Vacuum GmbH, D-50968 Köln), and VWR heated circulator bath, model # 1137. The system was kept at high vacuum 10^{-3} mPa and at a regulated temperature of 40 °C. The SAFE glass unit contained the dropping funnel where the solvent extract was placed after extraction (Figure 21).

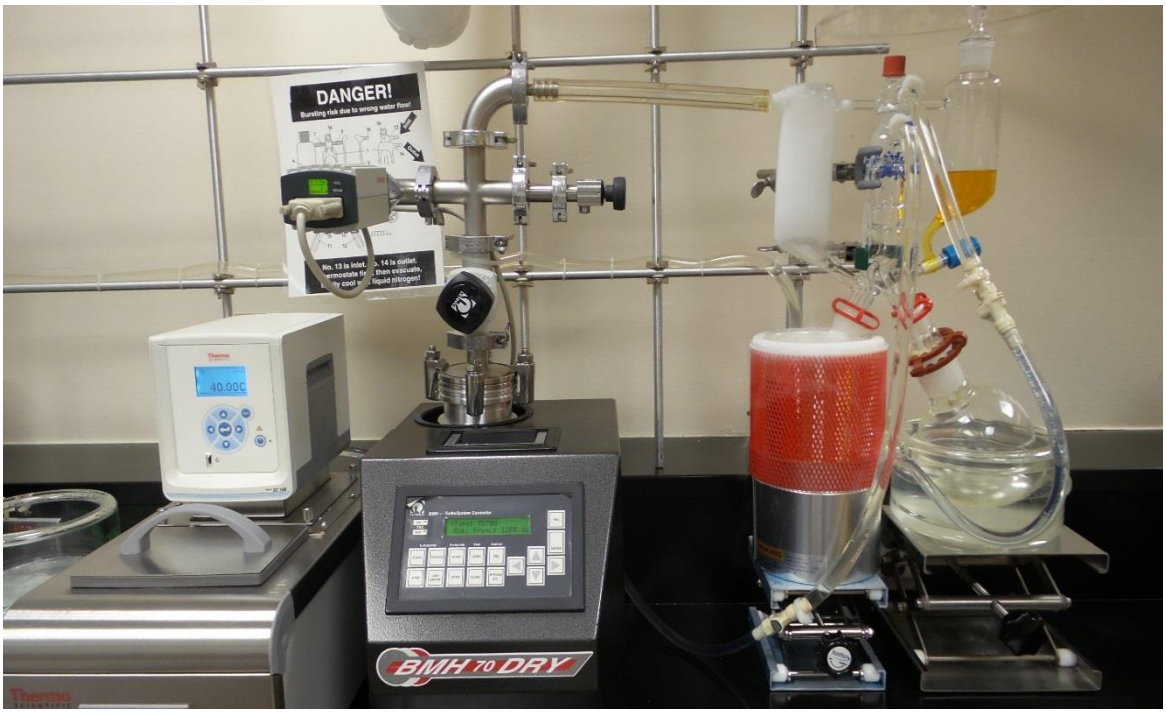

Figure 20. Solvent assisted flavor evaporation (SAFE) apparatus used for distillation of aroma compounds from the propolis samples.

The small amount of propolis (~5.5 g) was weighed into a plastic container, frozen with liquid nitrogen, then ground to a fine powder with a laboratory mill. The propolis powder (5 g) was transferred to a PTFE centrifuge tube. Freshly distilled diethyl ether (100 mL) was then added into the centrifuge tube containing the propolis powder. The propolis sample was extracted with diethyl ether at ambient temperature on an auto-shaker (Burrell Wrist Action Shaker) for 15 min. For separation of the solvent extract from the solid residue, the sample was centrifuged in a Sorvall RC 3C Plus centrifuge for 15 min at 4500 rpm.

SAFE apparatus was used to separate the volatile compounds from the non-volatiles of the propolis solvent extract. The organic fraction was placed in the SAFE apparatus dropping funnel and the extract was slowly dripped into the collecting flask (1000 mL) which was heated in a water bath at 40 °C. The volatiles including the solvent (diethyl ether) vapors were transferred through the central head and condensed in a collecting flask (500 mL) which was kept in a *Dewar* flask containing liquid nitrogen (Figure 21).

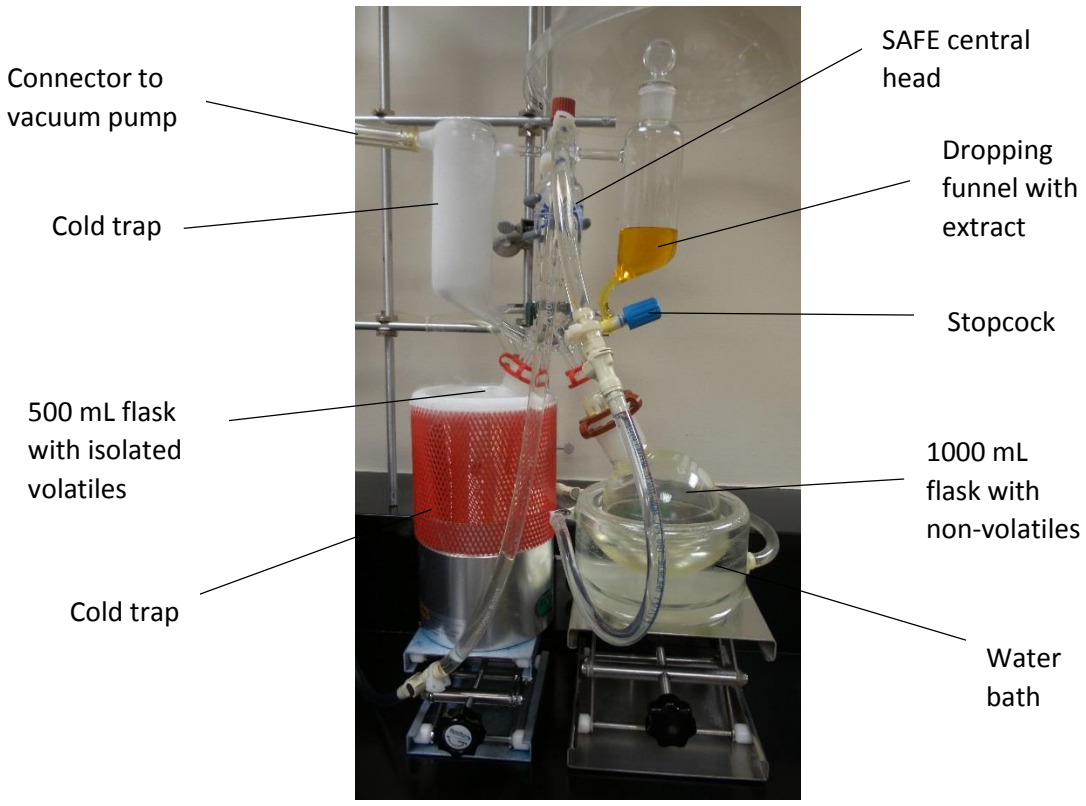

Figure 21. View of the assembled equipment for the SAFE technique.

The isolated SAFE volatiles were thawed at room temperature, dried over anhydrous sodium sulfate, filtered, and then concentrated to ~ 2 mL using a *Vigreux* column with a water bath held at 45 °C. Finally, the isolated volatiles were concentrated to ~ 200 µL with a gentle stream of nitrogen gas, and the concentrated mixture of volatiles was transferred to a GC vial with an insert for GC analysis.

6.6 Solid Phase Extraction (SPE)

An aroma isolate was prepared using the SAFE procedure described above, except with pentane as the solvent, in order to allow the polar volatiles to absorb to the silica. The SAFE isolate was concentrated on *Vigreux* column to ~1 mL and then loaded to a silica gel

SPE (2 g/12 mL Giga Tube, Strata SI-1 Silica (55 µm, 70A)) (Phenomenex, Torrance, CA) cartridge that had been sequentially conditioned with pentane, ether, and pentane (5 mL each). An aliquot of the sample (1 mL) was passed through silica and the cartridge then washed with solvents: pentane fraction A, pentane/ether (98:2 v:v) fraction B, pentane/ether (95:5 v:v) fraction C, pentane/ether (9:1 v:v) fraction D, pentane/ether (5:5 v:v) fraction E, ether fraction F (5 mL each). Increased ether concentration allowed the desorption of higher polarity compounds. Collected fractions (fraction A – F) were concentrated to ~ 200 µL under a gentle stream of nitrogen prior to GC analysis.

6.7 Gas Chromatography-Mass Spectrometry (GC-MS) to Identify Volatile Components

GC-MS analysis was performed on the propolis SAFE extract for compound separation and identification using an Agilent 6890 series gas chromatogram coupled to an Agilent 5973 mass spectrometer. The capillary column was fused silica GC column model HP-FFAP, 30 meter length, 0.32 millimeter inner diameter and 0.25 micrometer film thickness (30 m x 0.32 mm x 0.25 µm). An on-column injection of propolis extract (1 µL) was done by auto sampler with 10 µL syringe. Carrier gas was helium at a constant flow of 1 mL/min. With the GC in splitless mode, the injection temperature of 35 °C was held for 1 min at an initial pressure of 6.78 psi. The oven temperature was increased to 60 °C with a 60°C/min rate and then heated to 250 °C at 6°C/min rate and held at this temperature for 5 minutes. The total run time of the chromatographic analysis was 38.08 min. The mass spectrometry detector was coupled to GC via a transfer line heated at 250 °C. The mass spectrometer was operated in the electron impact (EI) ionization mode at 70 eV. The detector scan range was set to m/z 50 – 550.

6.8 Isolation of Volatiles by Purge and Trap Thermal Desorption (P&T-TD) and Identification of Components by Gas Chromatography Mass Spectrometry (GC-MS)

A Thermal Desorption Conditioning System (Scientific Instrument Services, Ringoes, NJ) was used for purge and trap collection of volatiles compounds. Prior to P&T, all three samples were crushed into a fine powder, and ~100 mg of each sample powder were measured into a 14 inch glass thermal desorption tube (0.5 inch O.D by 0.36inch I.D). Glass wool (0.5 g) was plugged into each glass tube from both ends. The tubes were placed in a sample collection oven and connected from one end with a stainless steel thermal desorption trap tube packed with Tenax ™ (6cm long x 3mm tube i.d.) as an absorbent trap, and the other end to a gas supply that purged nitrogen gas with a flow rate of 50 mL/min. Before desorption, the trap tubes were spiked with benzene-d6, toluene-d8, and naphthalene-d8 (1 µg each) (Sigma Aldrich, St. Louis, MO) as internal standards. The glass tubes were then incubated (100 °C) in sample collection oven for 30 min.

The traps were connected to a Short Path Thermal Desorption Unit, Model TD-2 (Scientific Instrument Services, Inc. Ringoes, NJ) located on the top of the GC injection port. The attached trap tube was initially purged with helium gas for 10 sec to flash the needle before injection. During thermal desorption the samples were thermally desorbed at 250 °C for 5 min with a 30 sec injection time.

A Varian 3400 GC system coupled to Finnigan MAT8230 double focusing magnetic sector MS was used for these analyses. The GC was equipped with ZB-5 capillary column (60m x 0.32mm x 1.0 µm film thickness) (Phenomenex, Torrance, CA). Helium was used as a carrier gas with the flow rate of 1.0 mL/min. The injection was done in a

split ratio of 10:1. The initial GC temperature (-20 °C) was held with dry ice for 5 min to insure cryofocusing, then the temperature was increased to 280 C° with a rate of 18 °C/ min. The GC-MS interface line was held at 280 C°. Mass spectrometer was in EI (70eV) mode with ion source temperature at 250°C. Scans were made from 35 m/z to 350 m/z, scan time was 0.6 s, and interscan time was 0.8 s.

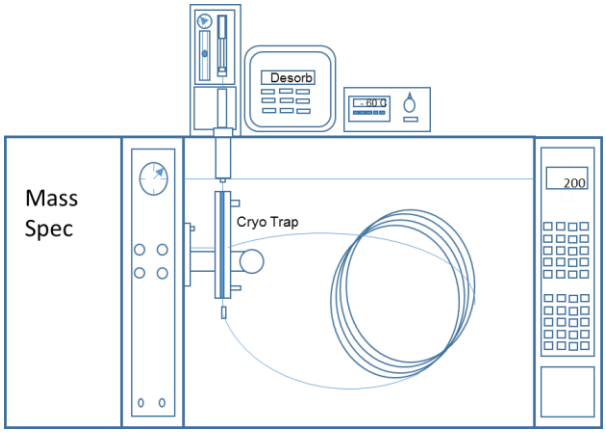

Figure 22. Purge and trap thermal desorption gas chromatography mass spectrometry diagram.

6.9 Compounds Identification

Compounds were identified from standard libraries using Chem Station and Mass Hunter software interfaced with the NIST library. Peak characterization was based on retention times from GC and molecular weights obtained from the MS. Reference standards were analyzed to confirm compounds identification. In addition hydrocarbon standards (HCS) C9 – C30 were analyzed on FFAP and ZB-5 column to confirm compound identities according to the retention indices (RI).

6.10 Identification of Odor-active Volatiles by Gas Chromatography-Olfactometry (GC-O)

Aroma-active compounds among the volatiles of propolis were distinguished by gas chromatography-olfactometry using an Agilent Technologies 6890 series Gas Chromatograph equipped with an HP-FFAP capillary column (30 m x 0.32 mm x 0.25 µm) and parallel FID detector and sniffing port. A 10 µL syringe was used to manually inject sample solvent extract (1 µL) at an initial temperature of 35 °C and initial pressure of 7.06 psi. Carrier gas was helium at a flow rate of 1.5 mL/min. Volatiles were separated using the following temperature program: hold initial temperature for a 1 min, increase to 60 °C at 60 °C/ min, increase to 240 °C at of 6 °C/ min, hold 10 min. A Y-type splitter at the end of capillary column divided the column effluent 1:1 into two 50 cm long paths going to the flame ionization detector (FID) and sniffing port, respectively. The FID detector was held at 250 °C with a hydrogen flow of 40.0 mL/min and air flow of 450 mL/min. The mode was set to a constant flow of 45 mL/min with helium gas. The custom-machined cylindrical cone (80 mm x 25 mm i.d.) was installed at the front of the FID detector. The sniffing port was heated to 180 °C.

6.11 Comparative Aroma Extract Dilution Analysis (cAEDA)

Comparative Aroma Extract Dilution Analysis (cAEDA) was employed for the characterization of the key odorants in propolis samples. The concentrated SAFE initial extract was first analyzed by injecting 1 µL of the sample to GC-O with an FFAP column to get the GC profile of the sample and to distinguish aroma-active compounds in the sample, then factor dilution (FD) were performed. The SAFE extract (FD1) was diluted

with solvent in 1:1 (v/v) ratio in then repeated steps. Each step thus represents a dilution of the sample, with 1024 being the highest tested. GC-O analysis was then conduced on each sample. The highest dilution at which each volatile could be detected was assigned as its flavor dilution (FD) factor. FD factors thus ranged from 1 to 1024 odorants with higher FD factors were consider to have the greatest contributions to the propolis aroma although this interpretation is recognized to have some limitations..

6.12 Microscopy Analysis

The microscopy analysis of the propolis samples were performed with an Olympus SZX12 (Olympus, Allentown, PA) microscope at a magnification of 15x for the visual description of the sample. Stream Essentials software was used for image acquisition. Photographs were taken with Olympus XC30 camera. Microscopic images were provided for visual purposes only.

7 Results and Discussion

7.1 Sensory Results

Sensory evaluation of propolis samples (3 in total) (Figure 6.1) collected over three consecutive seasons (year 1, year 2, and year 3) was conducted by trained panelists, using free-choice profiling (Table 6) and then a QDA (Figure 24). The aroma profiles were similar for each of the three years, all displaying typical propolis-like character. However, there were certain subtle differences detected from the different samples. The aroma profile of the propolis sample year 1 displayed a strong propolis-like aroma character with clove, honey, floral notes, and some cinnamon, eucalyptus, and piney notes. The aroma profile of propolis sample year 2 showed the mildest aroma intensity of all three years sampled. This aroma profile was characterized by a typical aroma character with clove, honey, and cinnamon notes, but less floral, eucalyptus, and piney notes. The aroma profile of propolis sample year 3 was similar to propolis sample year 1, displaying a strong propolis-like aroma character with clove, honey, floral notes, and some cinnamon, eucalyptus, and piney notes. In summary, all three years had similar aroma profiles with propolis years 1 and 3 being the most alike and propolis year 2 having the weakest aroma intensity.

Figure 23. Image of three propolis samples collected at the central NJ area over 3 consecutive years (Wolgast Tree Farm and Apiary) from the same bee hive.

Table 6. Sensory characterization of the three propolis samples by Orthonasal Free-Choice Profiling.

propolis	aroma description
Year 1	strong propolis aroma with clove, honey, floral notes and some cinnamon, eucalyptus, and piney notes
Year 2	mildest propolis aroma with clove, honey, and cinnamon notes and some floral, eucalyptus, and piney notes
Year 3	strong propolis aroma with clove, honey, cinnamon, floral, eucalyptus, and piney notes

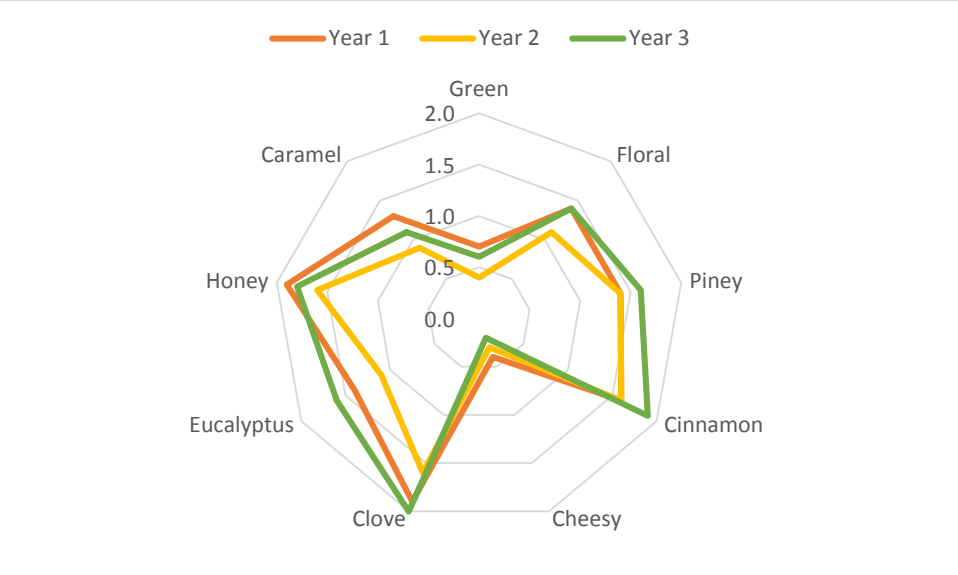

Figure 24. Spider plot representing quantitative descriptive analysis (QDA) results.

7.2 Screening Propolis for Aroma-Active Compounds

Propolis samples, collected over three consecutive seasons, were individually ground into fine powders, extracted with diethyl ether, and subjected to SAFE distillation. After distillation, the aroma volatile isolates were concentrated to about 200 µL prior to performing cAEDA. When evaluated sensorially, the aroma isolates smelled much like the authentic propolis samples and their sensory characteristics were found to be consistent with the results obtained for each year (years 1 through 3). Each propolis aroma isolate was subjected to a cAEDA, which resulted in a total of 47 aroma-active compounds in the FD factor range of 4 to 1024 (Table 7).

Mass spectra were acquired for 44 of the 47 compounds via GC-MS on FFAP column or P&T-TD-GC-MS on ZB-5 column (Table 7) (Figure 25 – 27).

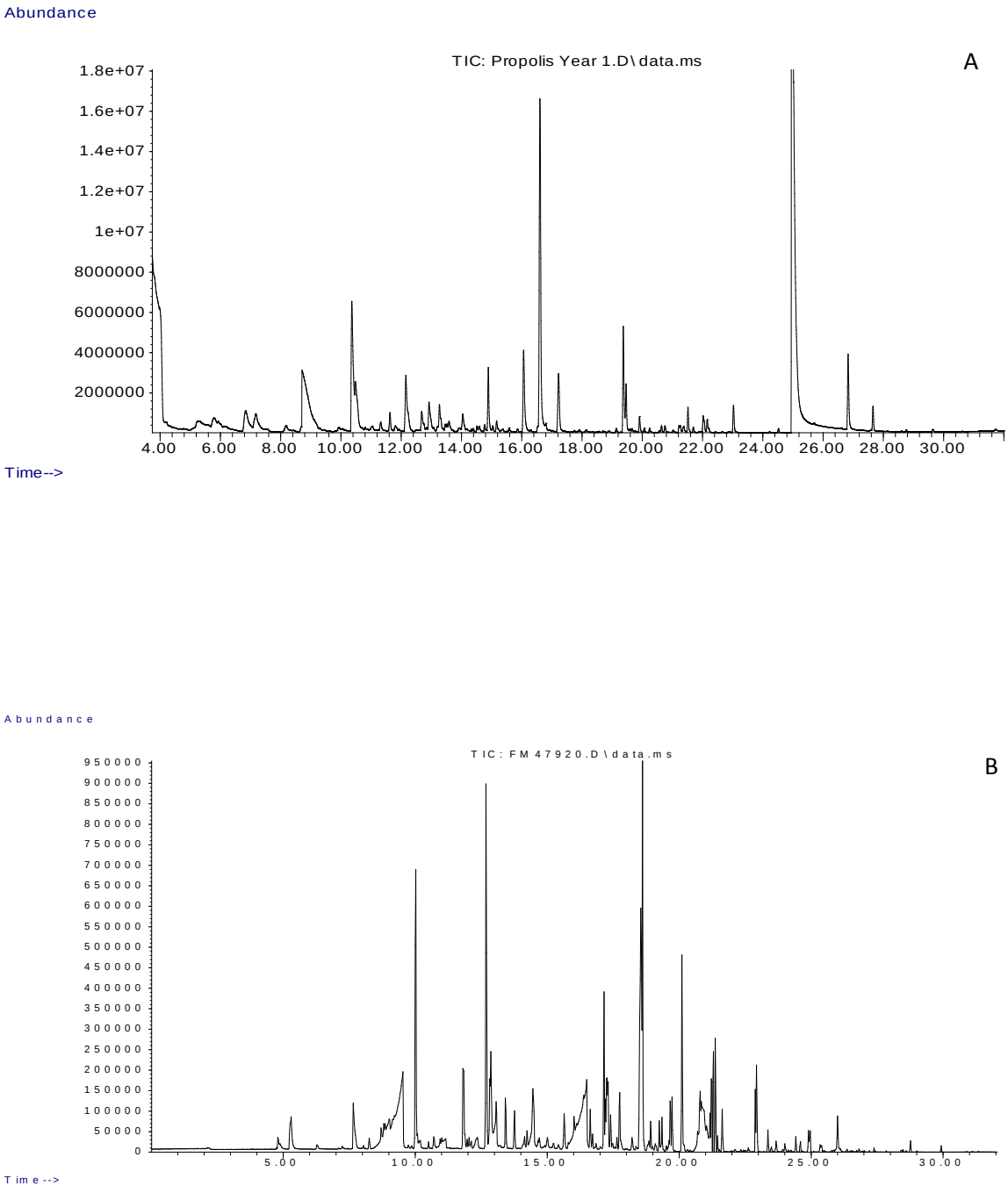

Figure 25. Total ion chromatogram of propolis year 1. A) GC-MS on FFAP column. B) P&T-TD-GC-MS on ZB-5 column.

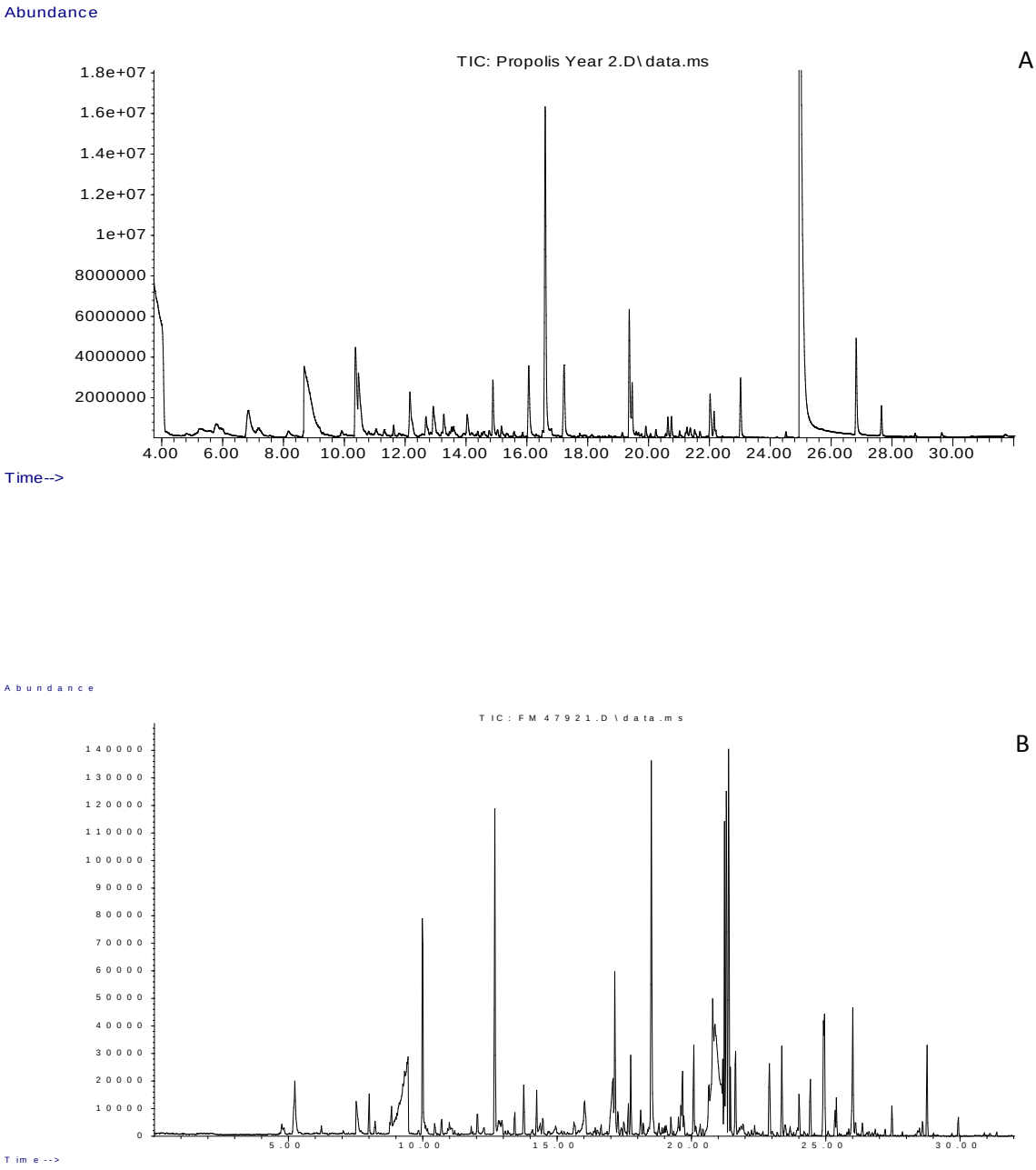

Figure 26. Total ion chromatogram of propolis year 2. A) GC-MS on FFAP column. B) P&T-TD-GC-MS on ZB-5 column.

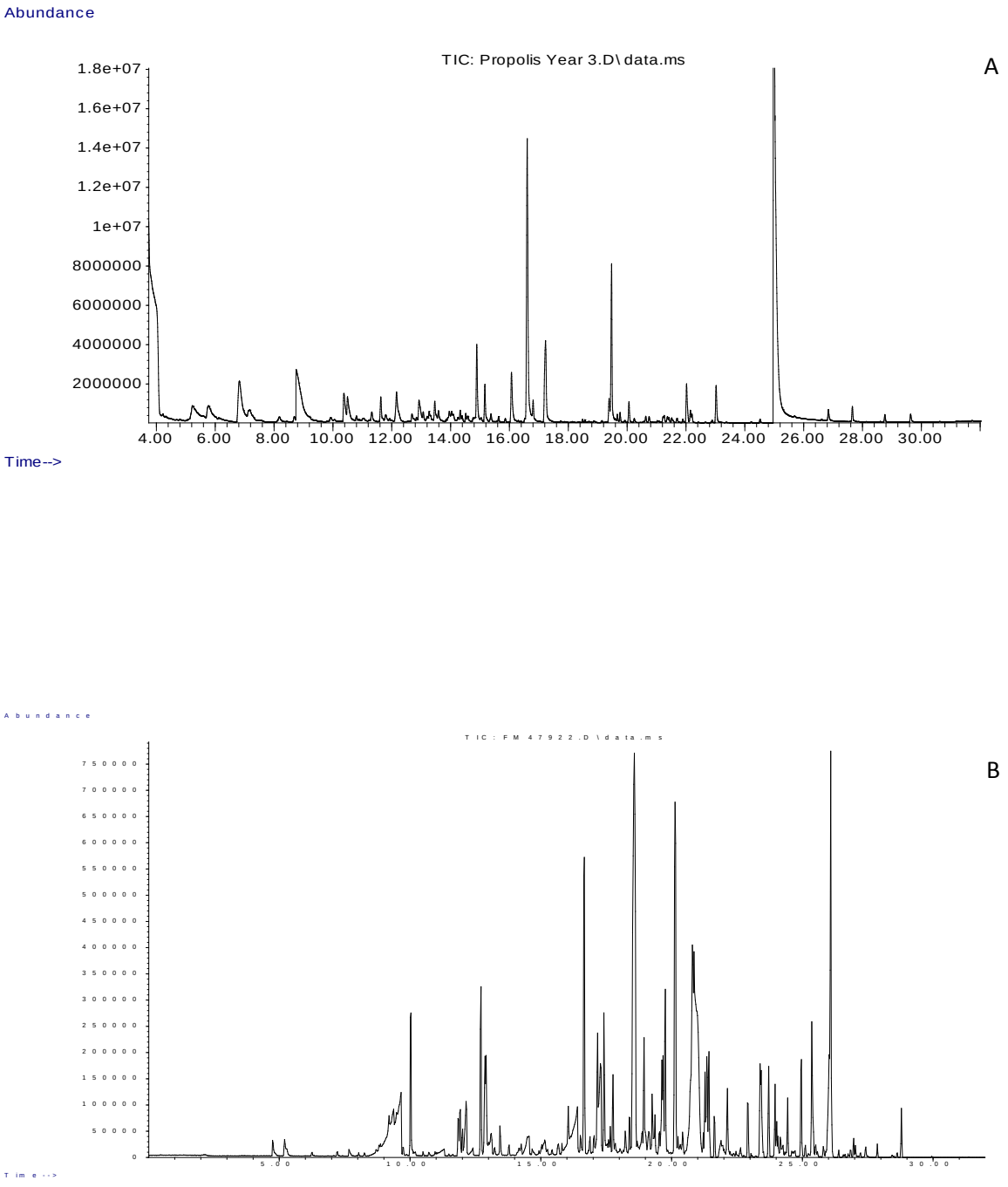

Figure 27. Total ion chromatogram of propolis year 3. A) GC-MS on FFAP column. B) P&T-TD-GC-MS on ZB-5 column.

Table 7. Aroma-active compounds (FD ≥ 4) in propolis samples collected over three consecutive years (year 1, year 2, and year 3).

			RI[d]		FD[e] factor				
no.[a]	odorant[b]	odor quality[c]	FFAP	ZB-5	Yr 1	Yr 2	Yr 3	fraction[f]	ref.[g]
1	hexanal[j]	green	1085	801	4		4	D	[17]
2	(E)-3-hexenal[j]	green	1130	792	16		16	E	[144]
3	α-pinene[j]	pine-like	1133	939	16	64	256	A	[21]
4	γ-terpinene	terpene-like	1185	979	4		16	A	[145]
5	1,8-cineole	eucalyptus	1194	1014	64	16	256	D	[145]
6	3-methylbutanal[j]	malty	1200	977	4	4	4	F	[17]
7	1-octen-3-one[h]	mushroom	1285	980	4		4		
8	dimethyl trisulfide	cabbage	1385	968	4	64	64		
9	acetic acid[j]	vinegar	1420	600	64	16	64	E	[17]
10	2,3- diethyl-5 methyl pyrazine[h]	earthy, nutty	1495	1158	4				
11	(E)-2-nonenal	green	1530	1161	256	256	256	E	
12	linalool[j]	floral	1550	1096	64	16	16	E	[145]
13	2-methylpropanoic acid[j]	sweaty	1565	1215	4	4	4	E	
14	(E,Z)-2,6-nonadienal	cucumber	1580	1150	256	64	256	E	[17]
15	butanoic acid[j]	sweaty, rancid, cheezy	1610	772	64	16	16	F	[17]
16	phenylacetaldehyde[j]	floral,honey	1639	1045	64	16	64	E	[146]
17	2- and 3- methylbutanoic acid[i,j]	sweaty	1661	885	64	16	16	F	[17]
18	(E,E)-2,4-nonadienal	fatty	1698	1212	64	4	16	E	
19	3-methyl-2,4-nonandione	hay-like	1715	1246	64	16	64	F	
20	phenylethyl acetate	floral	1785	1193			4	E	[17]
21	(E)-β-damascenone	cooked apple	1810	1384	256	64	64		
22	hexanoic acid	rancid	1840	973	64	16	16	F	[145]
23	2-methoxyphenol (guaiacol)	smoky	1860	1087	64	256	16	E	[79]
24	ethyl-3-phenylpropanoate	cinnamon	1888	1390	16	16	64		[146]
25	phenylethyl alcohol[j]	floral, rose	1901	1108	256	64	64	F	[147]
26	β-ionone	floral, berry	1980	1488	256	64	64		
27	δ-octalactone	coconut, creamy	1963	1278			16		
28	trans-4,5-epoxy-(E)-2-decenal[h]	metallic	2000	1380	4	4	4		
29	γ-nonalactone	coconut, creamy	2020	1361	16		16		
30	4-hydroxy-2,5-dimethyl-3(2H) furanone (HDMF)	caramel	2040	1080	16	16	16		
31	cinnamaldehyde[j]	cinnamon	2044	1267	256	256	64	E	
32	4-methoxybenzaldehyde[j]	anise-like	2050	1299	16	16	4	E	
33	methyl cinnamate[j]	cinnamon	2053	1300	64	16	64	E	[146]
34	cinnamyl formate	cinnamon	2065	1332	16	4	16	E	[147]
35	4-methylphenol	barnyard	2089	1178	4	4	4		
36	γ-decalactone	coconut, creamy	2125	1466	64	64	64		
37	(E)-ethylcinnamate	cinnamon	2130	1467	64	64	256	D	[146]
38	cinnamyl acetate	cinnamon	2144	1389	64	64	16	D	
39	eugenol[j]	clove	2177	1359	256	64	256	E	
40	4-ethyl phenol	phenolic	2168	1178	4	4	4	E	
41	2-methoxy-4-vinylphenol	clove	2248	1313	1024	1024	1024	E	[37]
42	2,6 dimethoxyl phenol	smoky	2271	1349	16	4	16		
43	cinnamyl alcohol[j]	floral	2284	1304	16	16	16	F	[147]
44	trans-isoeugenol	clove	2350	1451	16	64	64	E	[98]
45	phenylacetic acid	honey	2558	1274	16	4	16		[37]
46	4-hydroxy-3-methoxybenzaldehyde (vanillin)[j]	vanilla-like	2600	1394	64	64	16	F	[146]
47	3-phenylpropanoic acid	floral	2620	1343	256	64	256		[146]

Table 7. Caption

[a]Odorants were numbered according to their retention time on the FFAP column. [b]Identified by comparing the retention indices on the FFAP and ZB-5 column, the mass spectrum, as well as aroma quality and intensity with data obtained from authentic reference standards analyzed in parallel. [c]Odor quality as perceived during GC-O. [d]RI = linear retention index. [e]FD factor = flavor dilution factor. [f]Fraction in which the odorant was identified. [g]Reference of the compound as a propolis volatile. [h]Mass spectra could not be obtained in the propolis isolates. Identification was based on the remaining criteria as indicated above. [i]Odorants were not separated on either GC column. [j]Identified by P&T-TD-GC-MS.

The results of the cADEA and subsequent identification experiments resulted in a total of 13 odorants, shown in Table 8, as the strongest aroma-active compounds in the propolis samples, all of which exhibited an FD factor of ≥ 256 in a sample from at least one of the seasons. The highest FD factors were determined for 2-methoxy-4-vinylphenol (**41**) (clove-like), α-pinene (**3**) (pine-like), 1,8-cineole (**5**) (eucalyptus-like), (E)-2-nonenal (**11**) (green), (E,Z)-2,6-nonadienal (**14**) (cucumber-like), (E)-β-damascenone (**21**) (cooked apple), 2-methoxyphenol (**23**) (smoky), phenylethyl alcohol (**25**) (floral, rose), β-ionone (**26**) (floral, berry), eugenol (**39**) (clove-like), cinnamaldehyde (**31**) (cinnamon-like), (E)-ethyl cinnamate (**37**) (cinnamon-like), and 3-phenylpropanoic acid (**47**) (floral). The eight compounds (**3**, **5**, **14**, **23**, **25**, **37**, **41**, and **47**) have been previously identified in propolis [17, 21, 79, 145-147]. To the best of our current knowledge, compounds (**11**, **21**, **26**, **31**, and **39**) are reported here for the first time as aroma-active compounds in propolis (Figure 28).

Table 8. List of the key odorants of propolis which had an FD ≥ 256 during at least one of the three years tested.

			RI		FD factor		
no.	odorant	odor quality	FFAP	ZB-5	Yr 1	Yr 2	Yr 3
3	α-pinene	pine-like	1133	939	16	64	256
5	1,8-cineole	eucalyptus-like	1194	1014	64	16	256
11	(E)-2-nonenal	green	1530	1161	256	256	256
14	(E,Z)-2,6-nonadienal	cucumber	1580	1154	256	64	256
21	(E)-β-damascenone	cooked apple	1810	1384	256	64	64
23	2-methoxyphenol	smoky	1860	1087	64	256	16
25	phenylethyl alcohol	floral, rose	1901	1108	256	64	64
26	β-ionone	floral, berry	1980	1488	256	64	64
31	cinnamaldehyde	cinnamon	2044	1267	256	256	64
37	(E)-ethylcinnamate	cinnamon	2130	1467	64	64	256
39	eugenol	clove	2176	1359	256	64	256
41	2-methoxy-4-vinylphenol	clove	2248	1313	1024	1024	1024
47	3-phenylpropanoic acid	floral	2620	1343	256	64	256

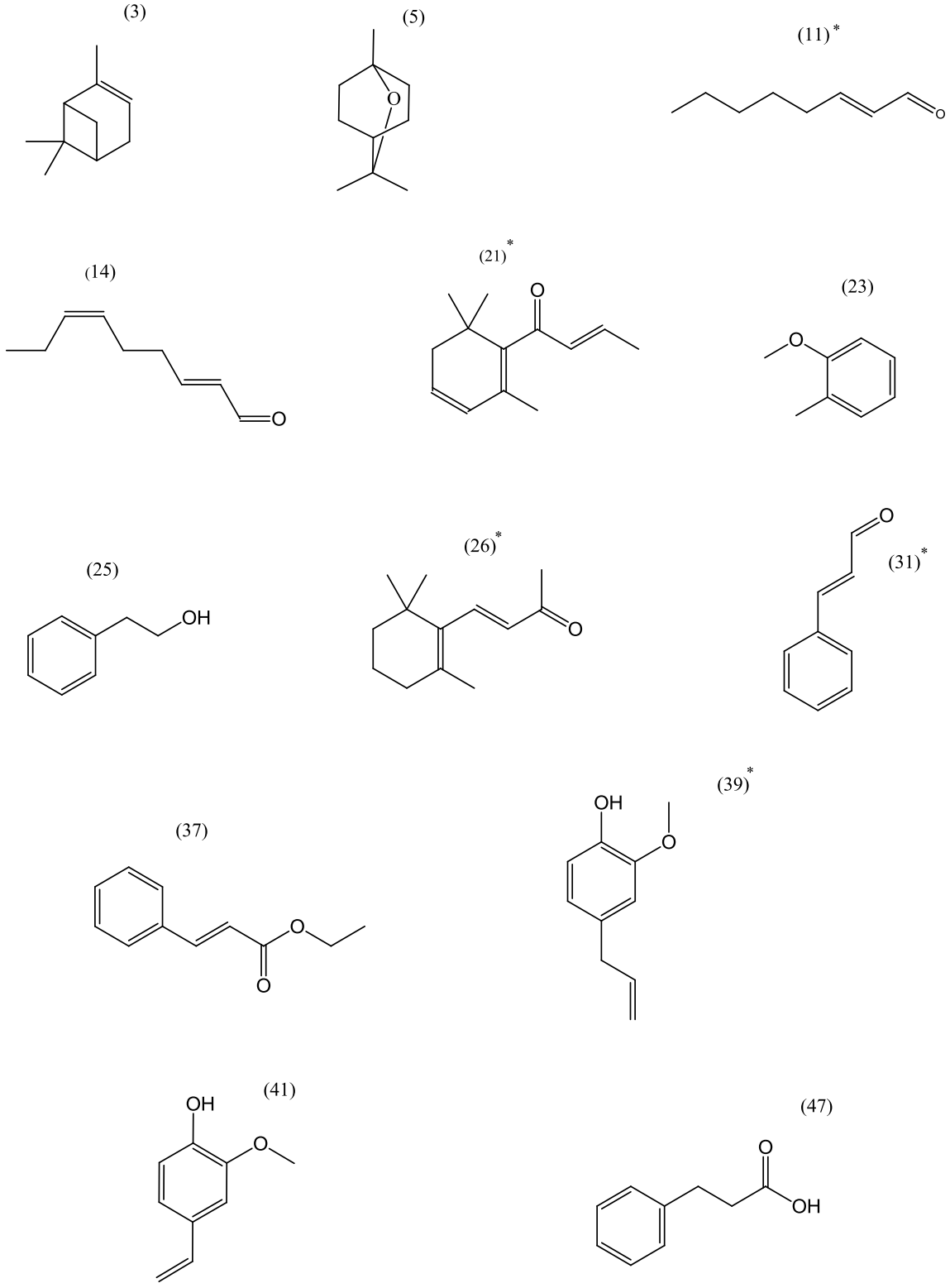

Figure 28. Structures of 13 key odorants of propolis year 1, year 2, and year 3 listed in Table 8. *indicates compounds identified as a key aroma active in propolis for the first time.

Among the odorants identified, clove-like smelling 2-methoxy-4-vinylphenol (**41**) displayed the highest FD factor (≥ 1024) in all three seasons. Thus, **41** appears to be one of the largest contributors to the characteristic aroma of the three samples analyzed. However, further studies are needed to support this hypothesis. To support this hypothesis, quantitation of **41** by stable isotope dilution assay (SIDA), calculation of odor activity values (OAV, ratio of concentration to odor threshold), aroma reconstitution experiments and omission tests may be conducted [148]. Silici and Kutluca previously reported the presence of **41** from propolis collected in East Anatolia by two different species of bees, namely, *Apis mellifera anatolica* and *Apis mellifera caucasica* [37]. In addition, Atac et al. reported the presence of **41** from Anatolian propolis. Although compound **41** was previously reported from propolis, to date it has not been described as a major aroma active compound from propolis. Recently, compound **41** had been identified as an important aroma active compound in rape honey [149].

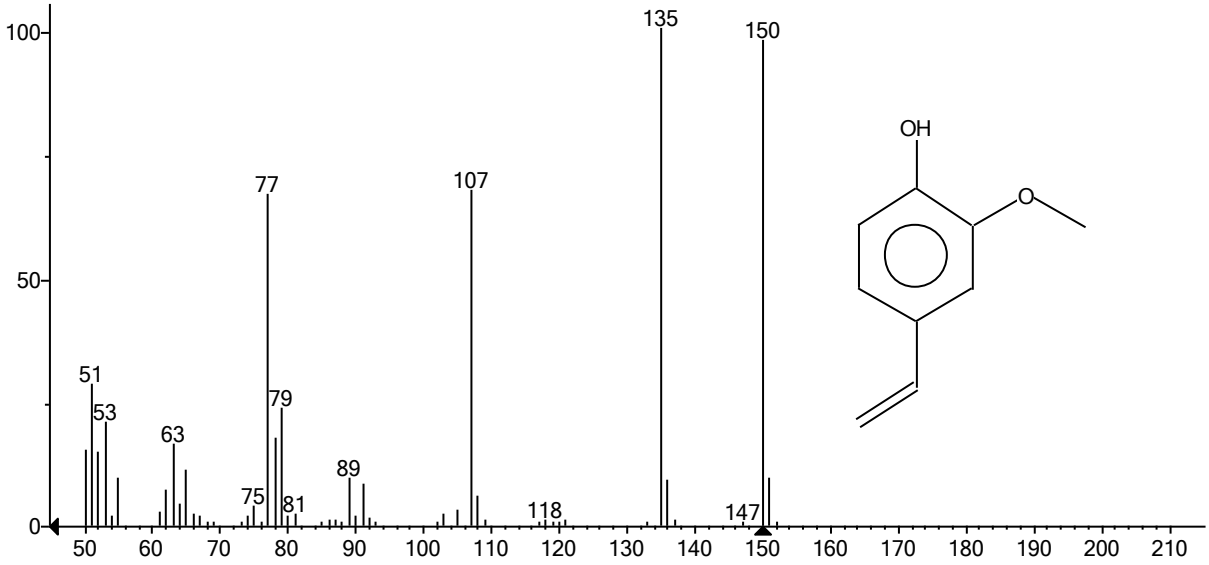

Figure 29. GC-MS mass spectrum of 2-methoxy4-vinylphenol (**41**).

To aid in the identification of some of the isolated volatile aromas, GC-O analysis provided some insight, but certain samples had either too low of an MS signal or were co-eluting with interfering compounds which made identification a challenge. To overcome this challenge, SAFE isolates prepared in pentene were fractionated by SPE and the resulting fractions were re-analyzed by GC-O and GC-MS. Three odorants, namely, 1-octen-3-one (**7**), 2,3- diethyl-5 methyl pyrazine (**10**), and trans-4,5-epoxy-(E)-2-decenal (**28**) occurred at too low a concentration in the propolis aroma isolates to acquire MS spectra; however, they were identified by analyzing the fractionated isolate by GC-O and comparing their aroma quality and intensity, and their RI (on both FFAP and ZB-5 columns) with that of reference standards (Table 7).

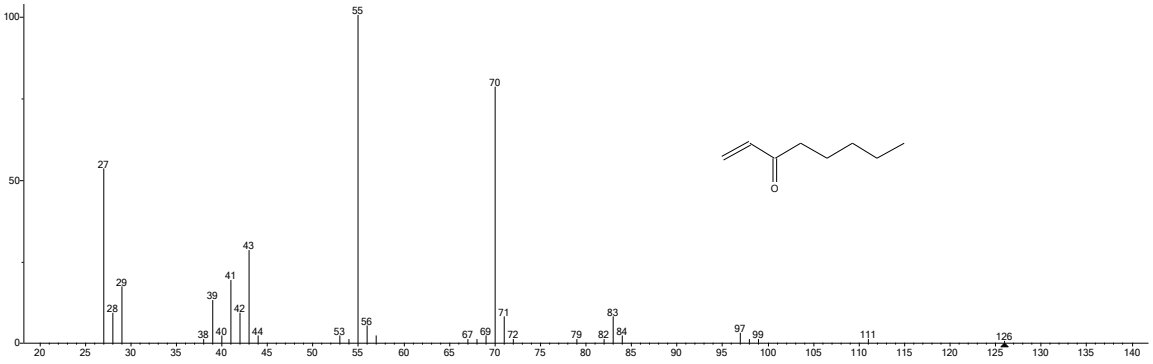

Figure 30. GC-MS mass spectrum of 1-octen-3-one (**7**) standard.

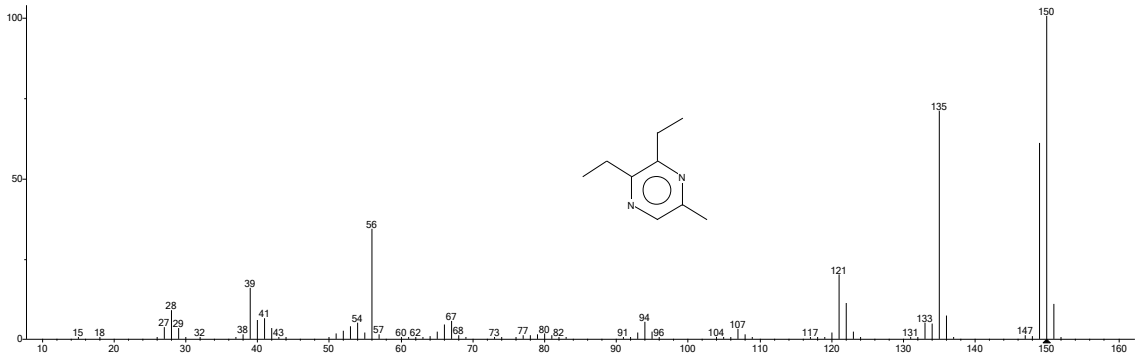

Figure 31. GC-MS mass spectrum of 2,3-diethyl-5-methyl pyrazine (**10**) standard.

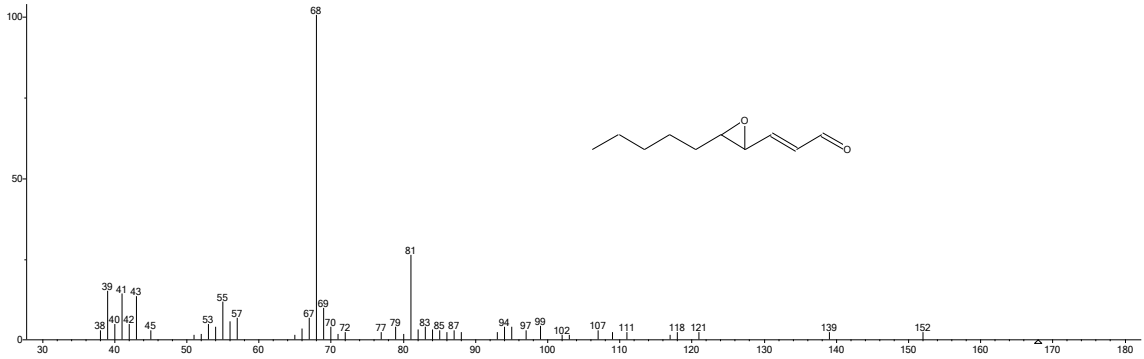

Figure 32. GC-MS mass spectrum of trans-4,5-epoxy-(E)-2-decenal (**28**) standard.

The sensory results from the aroma profiling exercise indicated that the aroma profile of propolis sample year 1 was found to be the most similar to the propolis sample year 3, in comparison to propolis year 2. Propolis from years 1 and 3 were higher in honey, clove, green and floral-like notes than propolis from year 2. The similar clove-like intensity of propolis samples from years 1 and 3 may be explained by similar FD factor for clove-like smelling eugenol (**39**, years 1 and 3; FD 256) as compared to year 2 (**39**, FD 64). Eugenol was identified from the propolis samples by both, volatile isolation techniques, SAFE and P&T-TD.

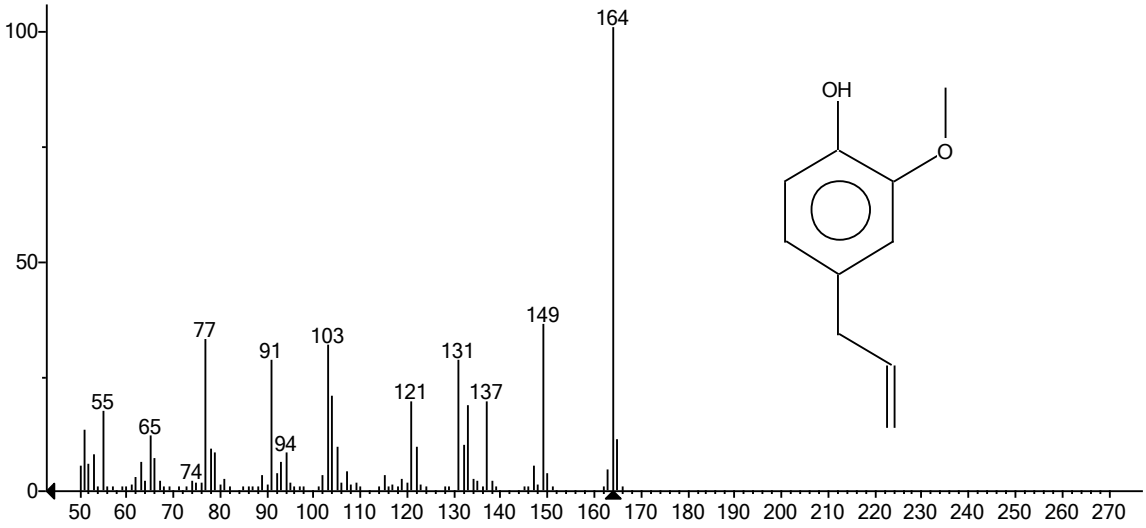

Figure 33. GC-MS mass spectrum of eugenol (**39**).

The similar honey-like intensity of the propolis samples from years 1 and 3 may be explained by similar FD factor for the floral/honey-like and honey-like smelling odorants, namely, phenylacetaldehyde (floral, honey-like) (**16**, years 1 and 3; FD 64) and phenylacetic acid (honey-like) (**45**, years 1 and 3; FD 16) as compared to year 2, (**16**, FD 16) and (**45**, FD 4), respectively. Compound **16** had been previously documented by Markham et al. in New Zealand propolis [146]. Silici et al. reported compound **45** in propolis from Turkey collected by *Apis mellifera carnica* bees [37].

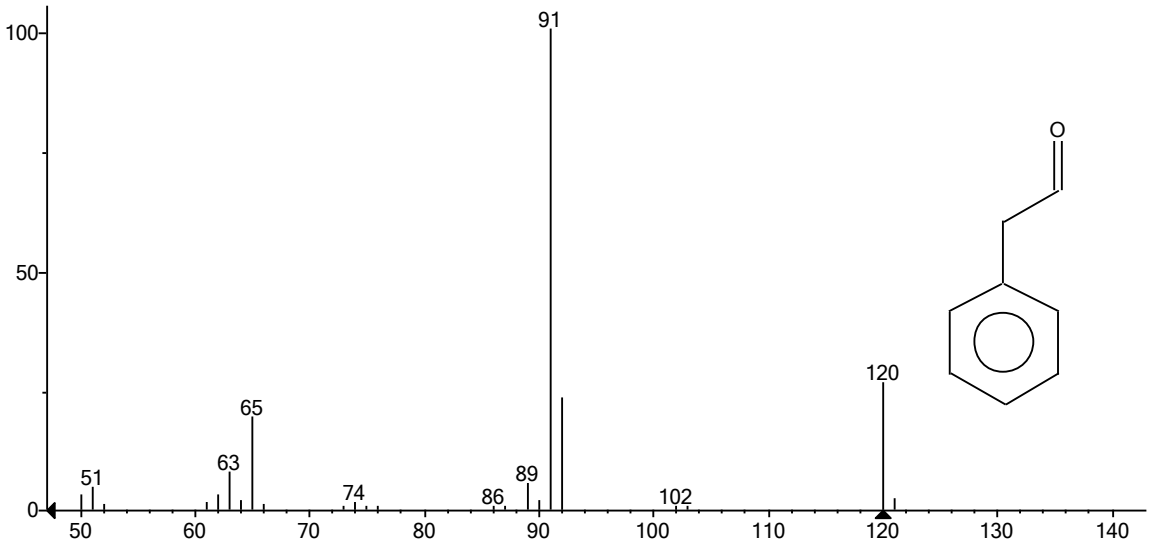

Figure 34. GC-MS mass spectrum of phenylacetaldehyde (**16**).

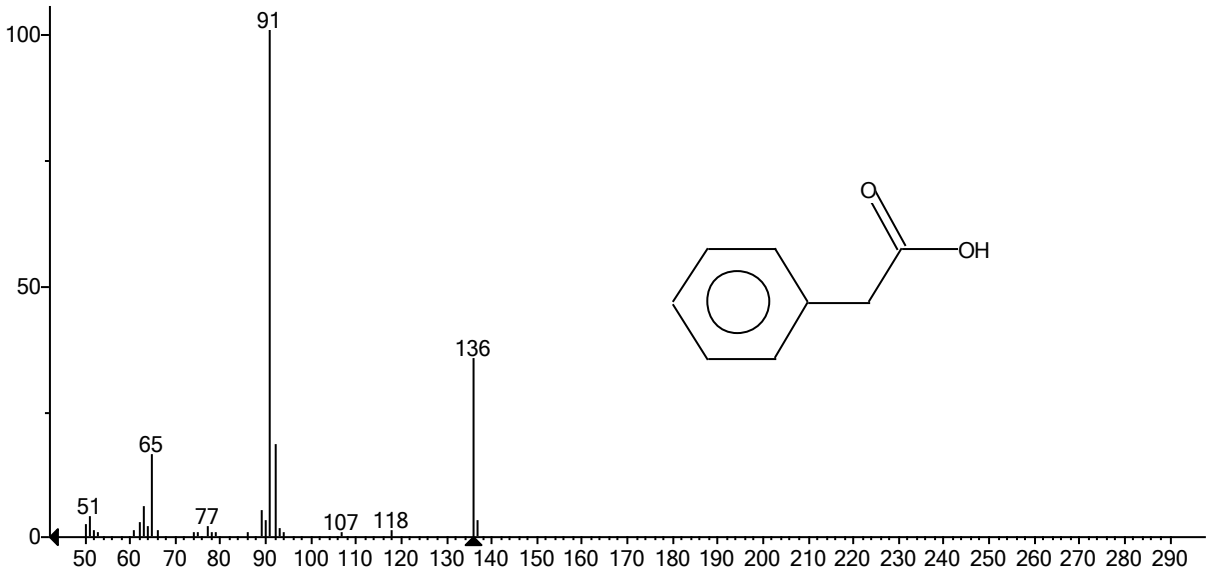

Figure 35. GC-MS mass spectrum of phenylacetic acid (**45**).

The similar floral sensory note of the propolis samples (years 1 and 3) may be explained by similar FD factors for the floral/honey-like and floral-like smelling odorants, namely, phenylacetaldehyde (Figure 34) (floral, honey-like) (**16**, years 1 and 3; FD 64) and 3-phenylpropanoic acid (floral) (**47**, years 1 and 3; FD 256) as compared to year 2, (**16**, FD 16) and (**47**, FD 64), respectively. Compound **47** had been previously reported by Markham et al. in propolis tincture solutions [146]. In addition to SAFE isolate analyzed on FFAP capillary column, the identification of phenylacetaldehyde was also confirmed by P&T-TD-GC-MS analysis on ZB-5 column.

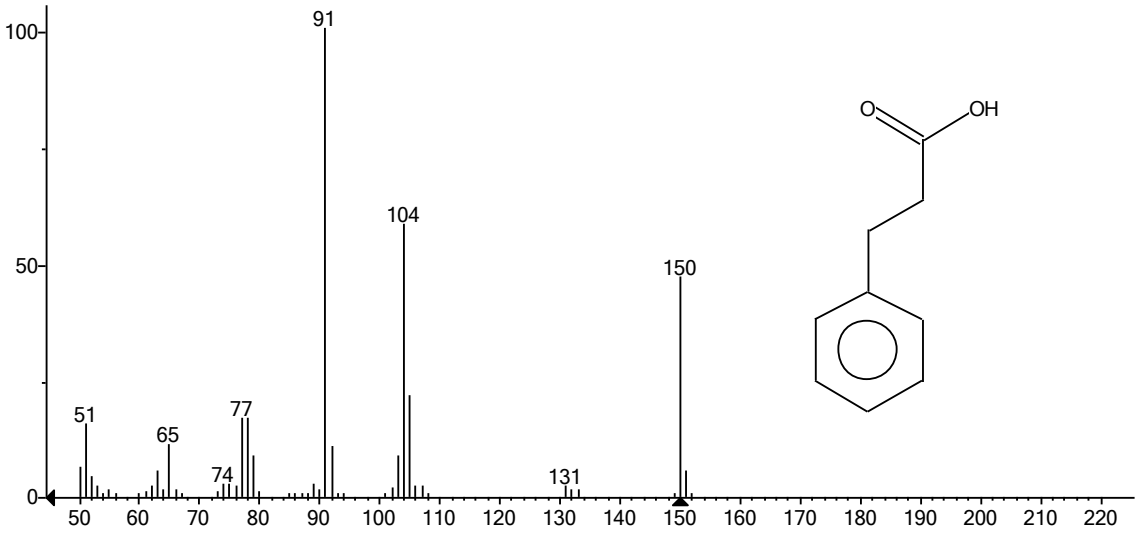

Figure 36. GC-MS mass spectrum of 3-phenylpropanoic acid (**47**).

Although propolis samples from year 1 and 3 were similar in aroma profile, propolis from year 3 had higher intensities of piney, eucalyptus, and cinnamon-like notes than propolis from year 1. The higher intensity of piney notes in propolis year 3 as compared to propolis years 1 and 2 may be explained by the higher FD factor of the piney smelling odorant, α-pinene (**3**, FD 256) in the propolis year 3 sample. Melliou et al. previously documented the presence of α-pinene in Greek propolis [21]. Aroma isolations by SAFE and P&T-TD confirmed the presence of α-pinene in propolis samples from all three years.

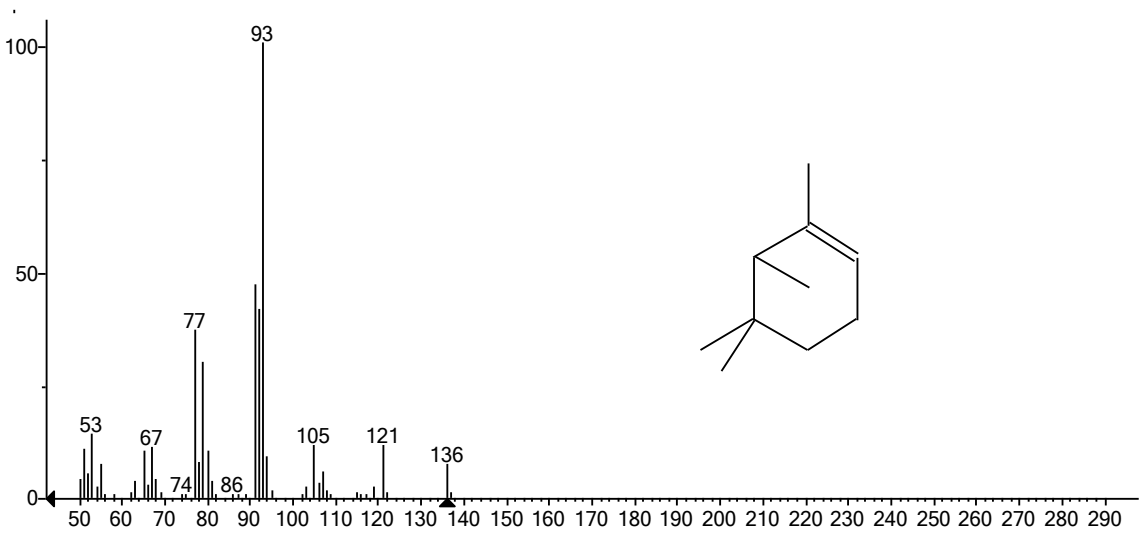

Figure 37. GC-MS mass spectrum of α-pinene (**3**).

The higher intensity of the eucalyptus-like note in propolis year 3 may be explained by the higher FD factor for the eucalyptus-like smelling odorant, 1,8-cineole (**5**, FD 256) in the sample. Compound **5** was reported as a principal component in Brazilian propolis (Piaui region) by Torres et al. [145].

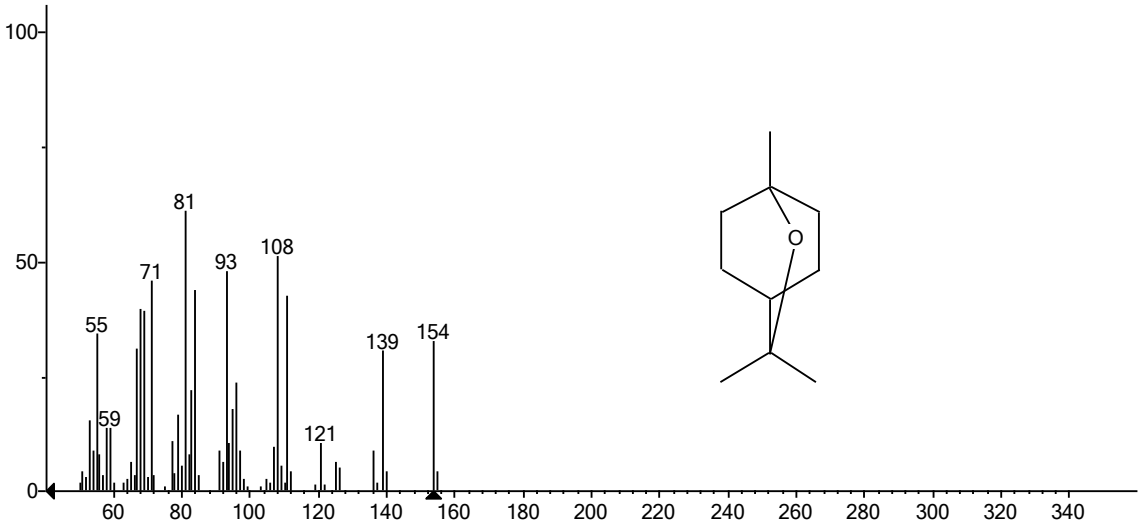

Figure 38. GC-MS mass spectrum of 1,8- cineole (**5**).

The higher intensity of the cinnamon-like note may be explained by the higher FD factor for the two cinnamon-like smelling odorants, (E)-ethyl cinnamate (**37**, FD 256) and ethyl-3-phenylpropanoate (**24**, FD 64) in the year 3 sample. **37** and **24** were previously documented as propolis volatile compounds [79, 146].

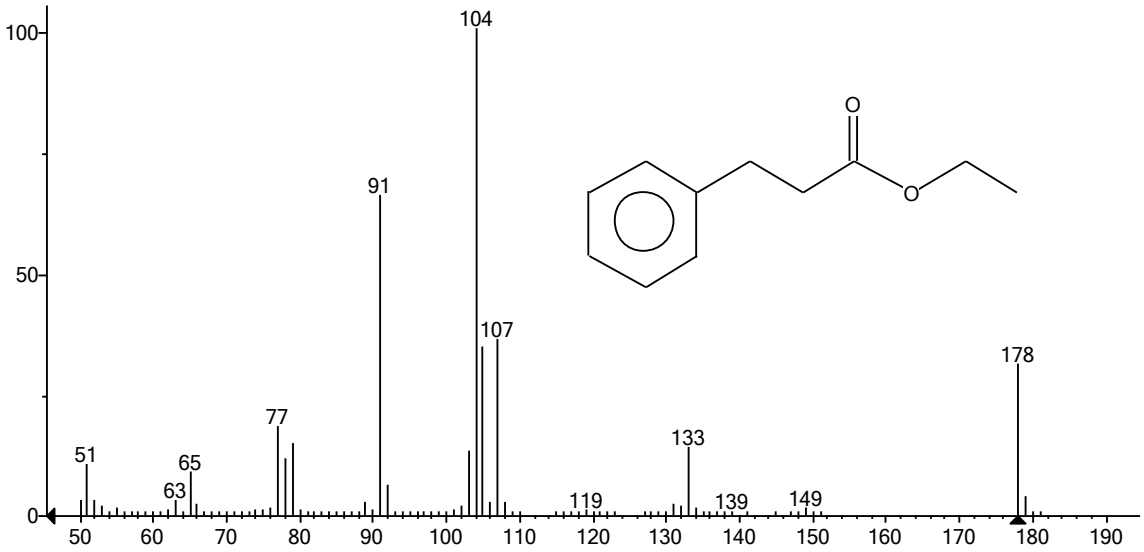

Figure 39. GC-MS mass spectrum of ethyl-3-phenylpropanoate (**24**).

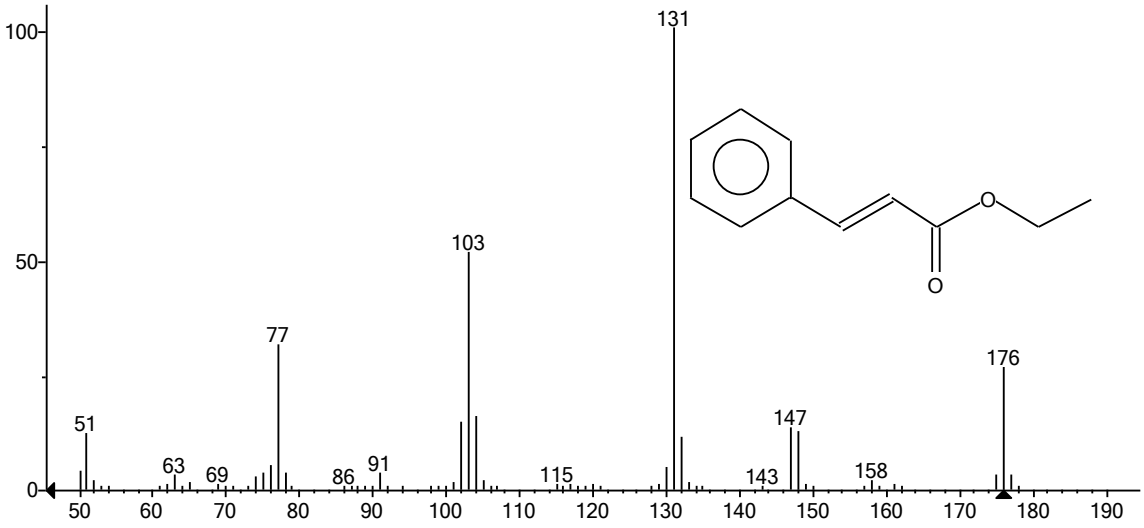

Figure 40. GC-MS mass spectrum of (E)-ethylcinnamate (**37**).

Although the overall difference between years were small, some of the sensory differences noted in this study may be explained by the variable FD factors observed for selected odorants during the cAEDA (Figure 41 – 43).

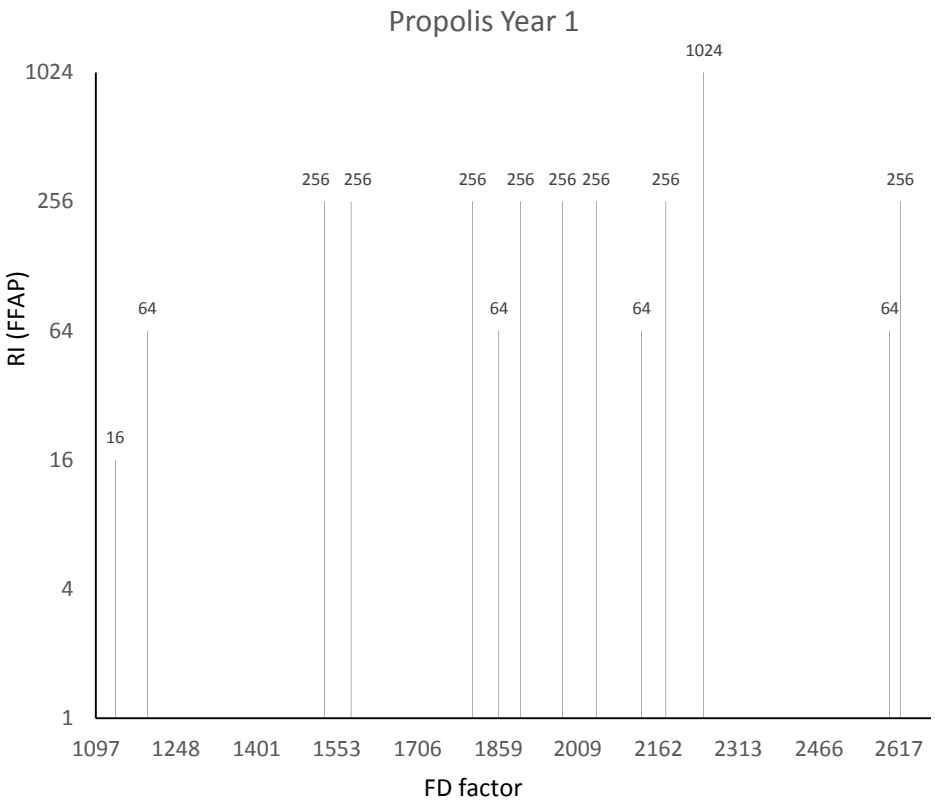

Figure 41. Aromagram representing key odorants of propolis Year 1 on FFAP column (FD ≥ 16).

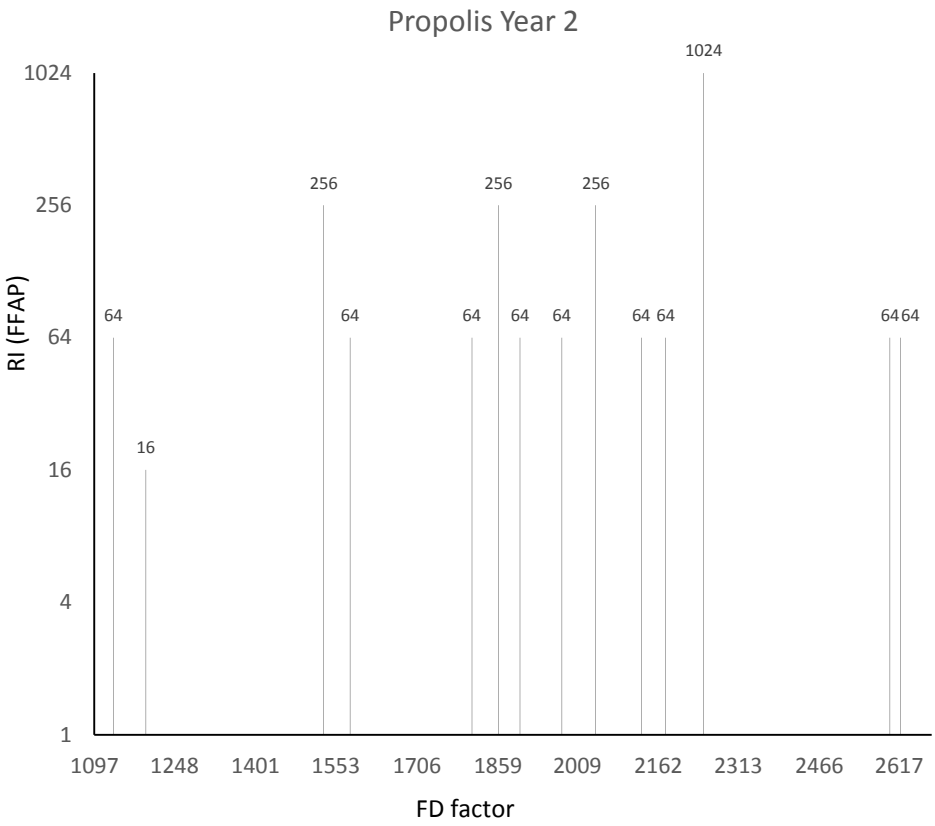

Figure 42. Aromagram representing key odorants of propolis Year 2 on FFAP column (FD ≥ 16).

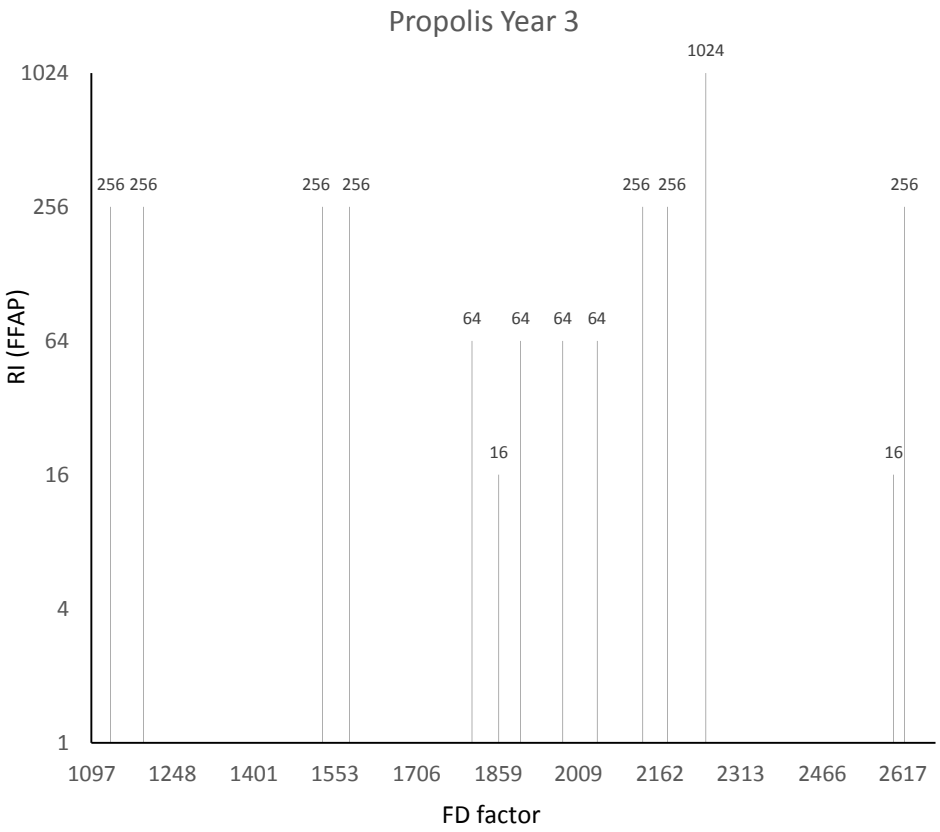

Figure 43. Aromagram representing key odorants of propolis Year 3 on FFAP column (FD ≥ 16).

The earthy smelling odorant 2,3-diethyl-5 methyl pyrazine (**10**) was detected only in propolis from year 1, while the coconut smelling and floral/honey-like smelling odorants, δ-octalactone (**27**) and phenyl ethyl acetate (**20**), respectively, were detected only in propolis from year 3. To the best of our current knowledge, this is the first report of compounds **10** and **27** as aroma active compounds identified in propolis.

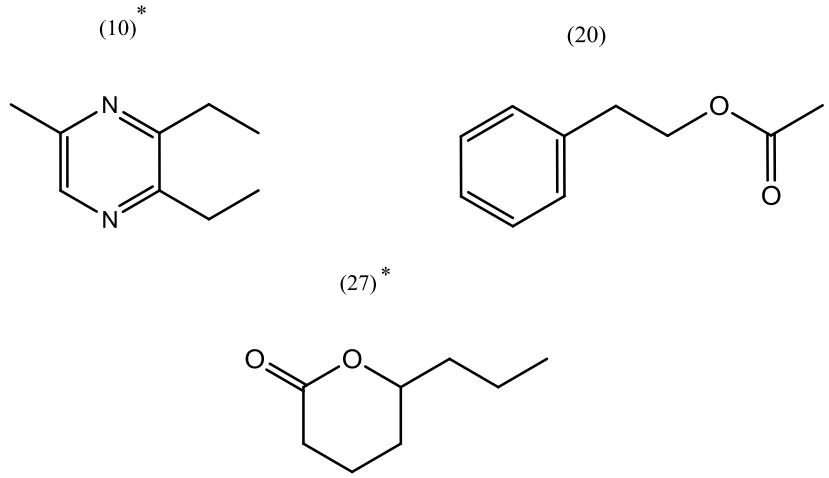

Figure 44. Structures of 2,3-diethyl-5-methyl pyrazine (**10**) only identified in propolis Year 1 and phenyl ethyl acetate (**20**), and δ-octalactone (**27**) only identified in propolis Year 3. *indicates compounds identified as a key aroma active in propolis for the first time.

In addition, the five odorants, namely, hexanal (**1**), (E)-3-hexenal (**2**), γ-terpinene (**4**), 1-octen-3-one (**7**), and γ-nonalactone (**29**) were detected only in propolis from years 1 and 3, and not detected in propolis year 2. Nevertheless, the propolis samples were remarkably consistent over the three consecutive seasons, resulting in a total of 39 odorants detected in all three years of which only some FD values differed amongst the three seasons (Table 7). Compound (**1**) was extracted by both SAFE and P&T-TD technique, however compounds (**2**), (**4**), (**7**), and (**29**) were only detected by SAFE in combination with GC-MS analysis on FFAP and ZB-5 capillary columns.

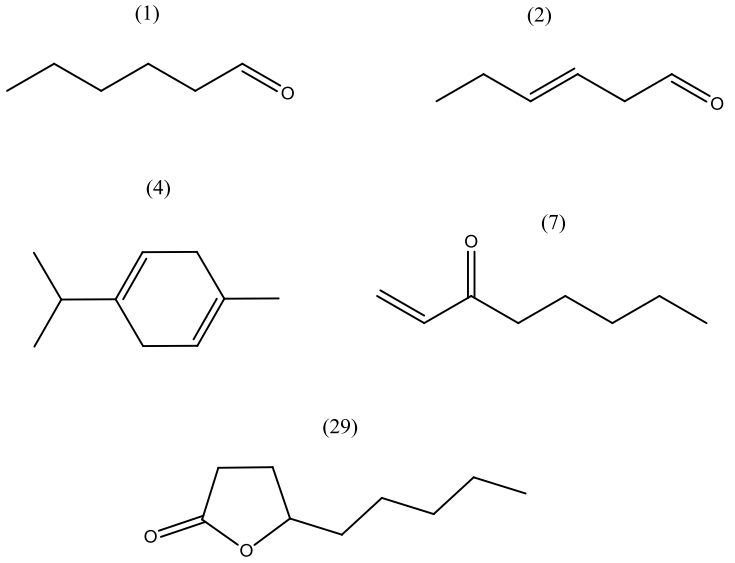

Figure 45. Structures of hexanal (**1**), (E)-3-hexenal (**2**), γ-terpinene (**4**), 1-octen-3-one (**7**) and γ-nonalactone (**29**) identified only in propolis Year 1 and 2, not in Year 3.

Three key odorants (E)-β-damascenone (**21**) (cooked apple), phenyl ethyl alcohol (**25**) (floral, rose), and β-ionone (**26**) (floral, berry) shows similar FD factors in propolis over three consecutive years. In year 1, compounds **21**, **25** and **26** had FD factor of 256, and for years 2 and 3 the FD factor was 64.

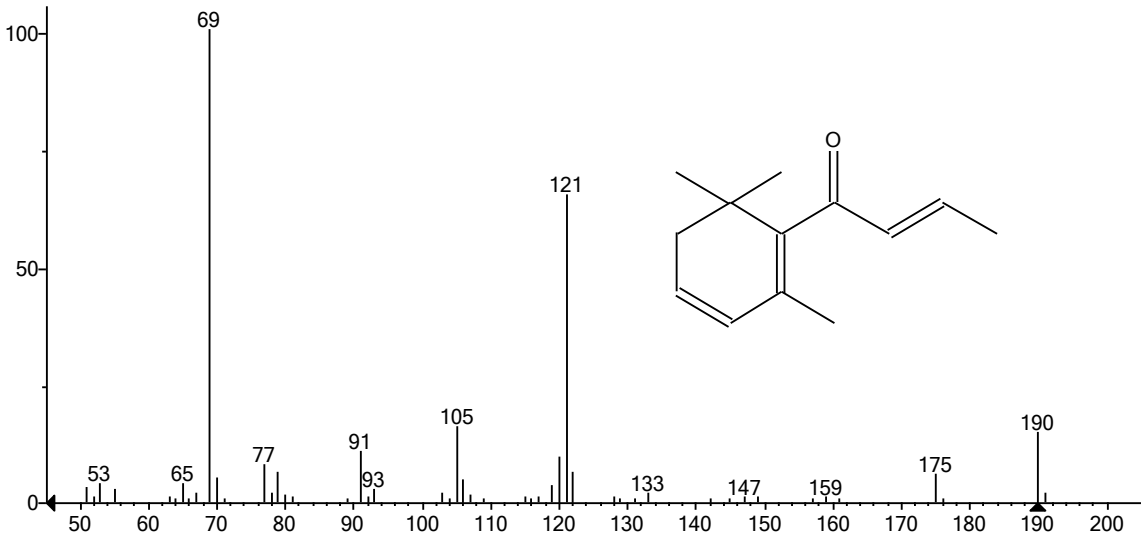

Figure 46. GC-MS mass spectrum of β-damascenone (**21**).

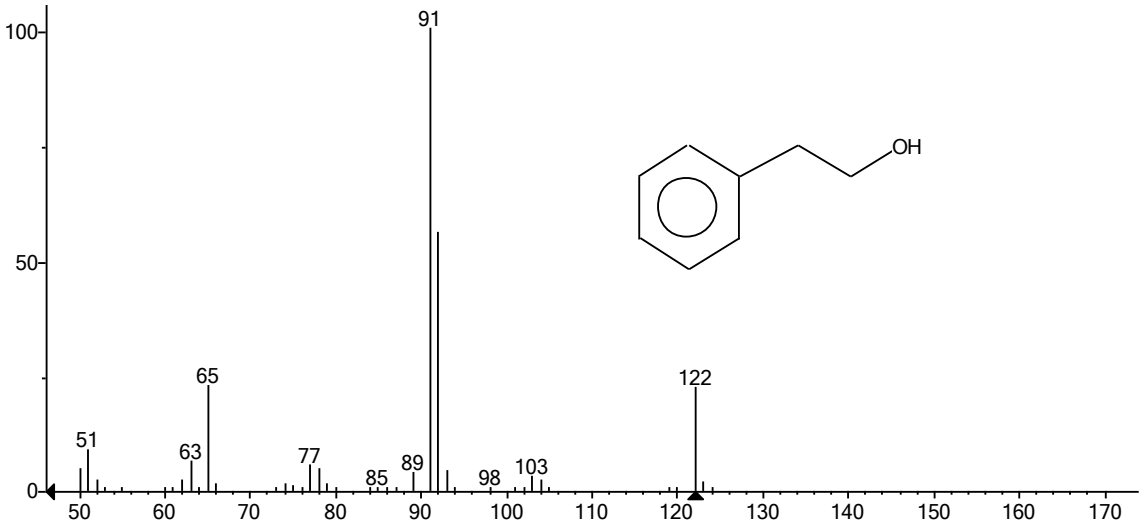

Figure 47. GC-MS mass spectrum of phenylethyl alcohol (**25**).

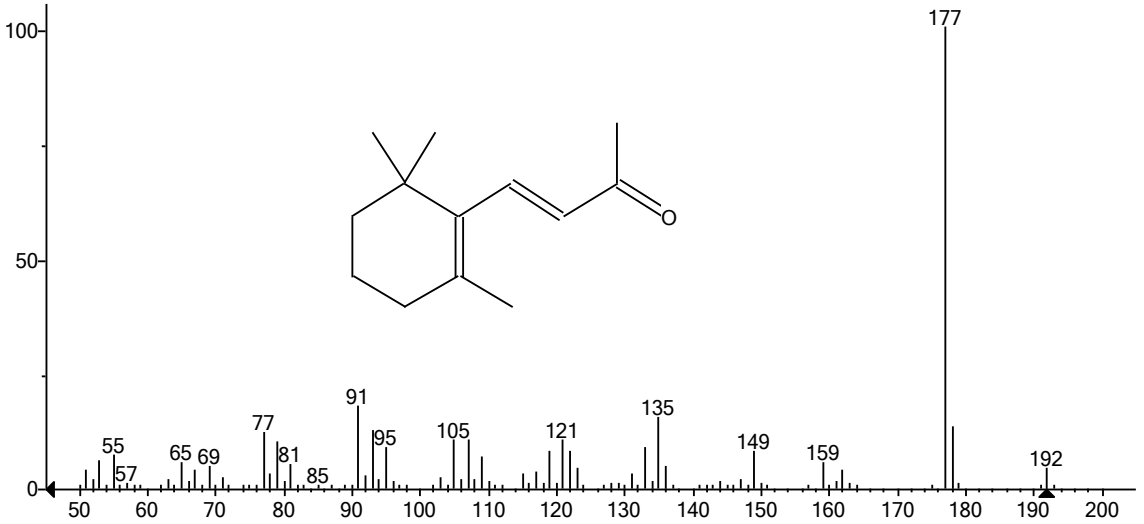

Figure 48. GC-MS mass spectrum of β-ionone (**26**).

The green smelling odorant (E)-2-nonenal (**11**) appears to be the second highest contributor to propolis aroma throughout years 1, 2 and 3. Sensory QDA test shows the differences in green note in all three samples. Alike to 2-methoxy-4-vinylphenol (**41**, FD 1024), (E)-2-nonenal (**11**, FD 256) has a consistent intensity over all three years. The presence of (E,Z)-2,6-nonadienal (**14**, cucumber, FD 256 year 1 and FD 64 year 2) may also influence the perception of the green note in the samples, due to its cucumber-like aroma quality.

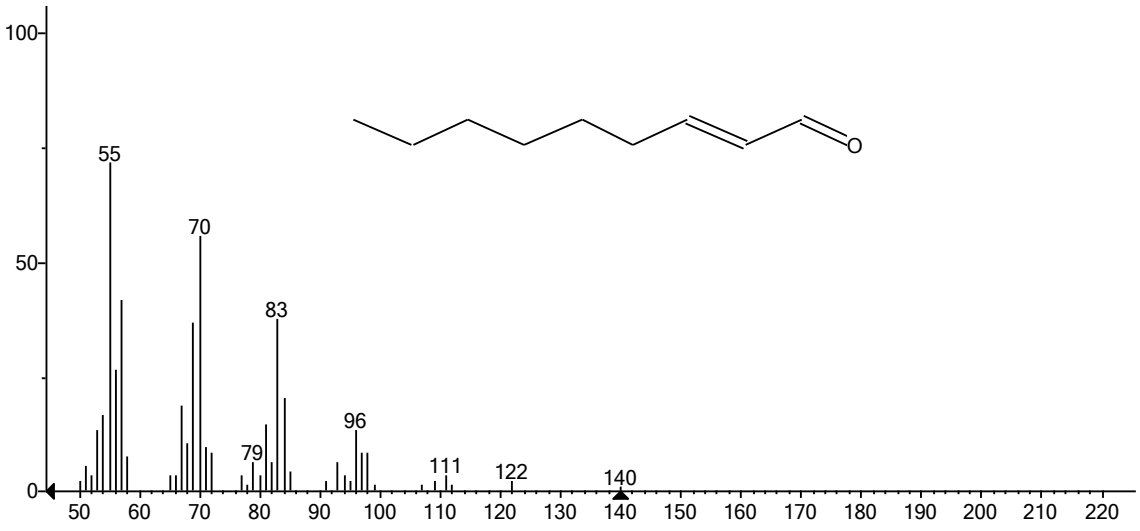

Figure 49. GC-MS mass spectrum of (E)-2-nonenal (**11**).

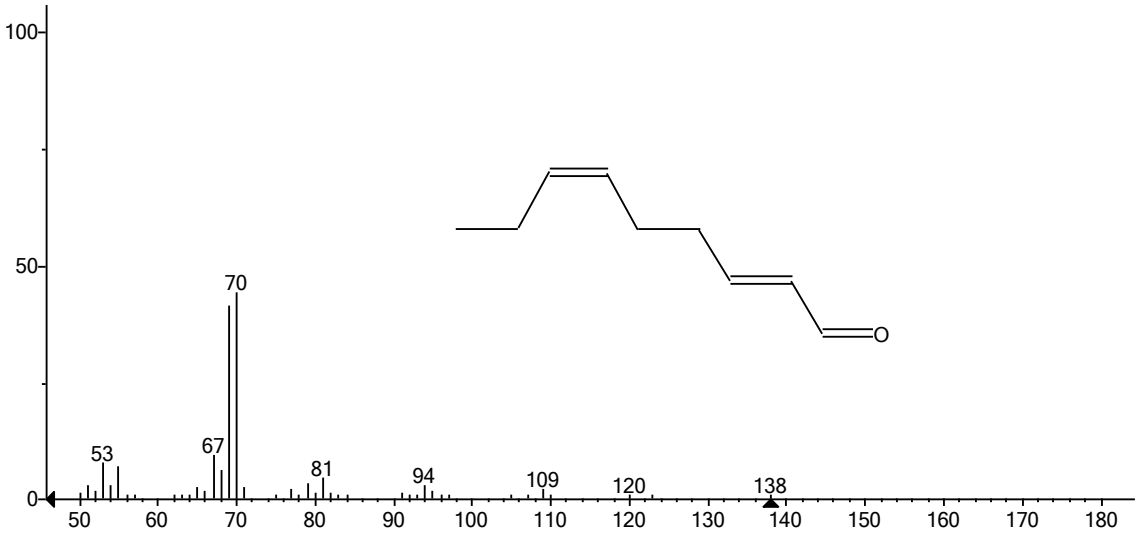

Figure 50. GC-MS mass spectrum of (E,Z)-2,6-nonadienal (**14**).

The lower intensity of 2-methoxyphenol (**23**) which contributes a smoky note and cinnamaldehyde (**31**) with cinnamon note was observable in propolis year 3. Compound **23** (FD 16, year 3) has a lower intensity than in propolis year 1 (FD 64) and propolis year 2 (FD 256). Also, compound **31** (FD 64, year 3) had a lower intensity than in propolis year 1 and 2 (FD 256).

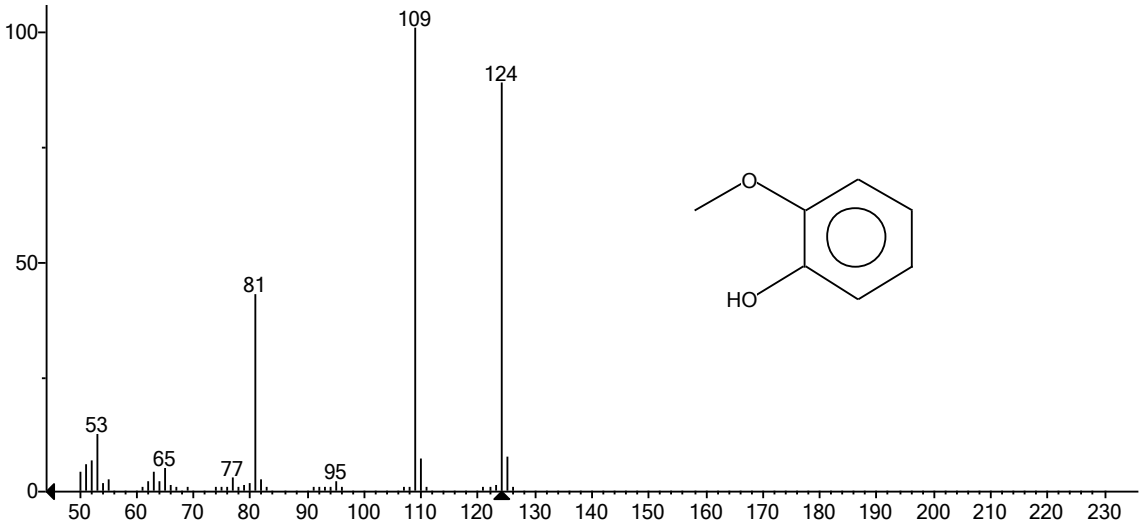

Figure 51. GC-MS mass spectrum of 2-methoxyphenol (**23**).

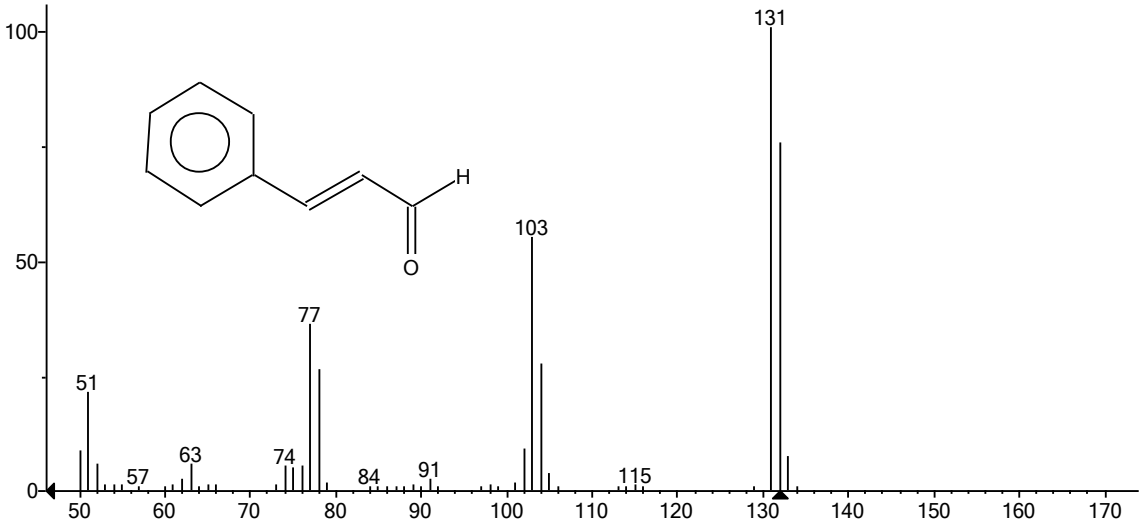

Figure 52. GC-MS mass spectrum of cinnamylaldehyde (**31**).

Of the total 47 odorants identified in the propolis samples, 39 were detected in all propolis samples (years 1 through 3). Only 8 of the 47 total odorants were not detected in all 3 years. The following compounds, namely, hexanal (**1**), (E)-3-hexenal (**2**), γ-terpinene (**4**), 1-octen-3-one (**7**), 2,3-diethyl-5-methyl pyrazine (**10**), phenylethyl acetate (**20**), δ-octalactone (**27**), and γ-nonalactone (**29**) were not detected in at least one of the samples. It is not surprising that these compounds were not detected in some of the samples due to the fact that propolis is a natural product that is variable and collected from various plant sources [8].

The formation of aroma compounds in propolis can be characterized and explained by different reaction pathways but not limited to, lignin pyrolysis, lipid oxidation, and Maillard reaction which includes Strecker degradation.

Aroma compounds generated via lignin pyrolysis are formed by the thermal degradation or decomposition of phenolic acids and lignin by microorganisms into phenols [140]. The following phenols were found in propolis and they are 4-ethylphenol (phenolic), 2-methoxy-4-vinylphenol (clove), 2,6-dimethoxyphenol (smoky), eugenol (clove) and trans-isoeugenol (clove). As a result of the pyrolysis of ferulic acid, 2-methoxy-4-vinylphenol (clove) was the main product formed however secondary products such as 4-hydroxy-3-methoxybenzaldehyde (vanilla) and 2-methoxyphenol (smoky) were also generated.

The majority of propolis odorants were generated during lipid peroxidation and lipoxygenase catalysis. The autoxidation of oleic, linoleic and linolenic acid can lead to the formation of aldehydes and ketones. Linoleic acid, a compound present in lipids that is very sensitive to autoxidation, is a precursor of hexanal. There are also a number of

additional aldehydes that can be formed during the degradation of fatty acids such as (E,Z)-2,6-nonadienal (cucumber) and (E,E)-2,4-nonadienal (fatty).

Aroma compounds produced via Maillard reaction, which is the reaction between an amino acid and a reducing sugar, are as followed: 4-hydroxy-2,5-dimethyl-3(2H)furanone (caramel) and 2,3-diethyl-5-methylpyrazine (earthy, nutty). Strecker degradation products, which are formed between deoxyosones or dicarbonyl compounds formed during carbohydrate degradation reacting with a free amino acids, were also generated and they are as followed: 3-methylbutanal (malty) and phenylacetaldehyde (floral, honey).

Additional aroma compounds present in propolis can be characterized according to following compound classes: lactones, monoterpenes, sesquiterpenes, carotenoid-derived compounds such as α- and β-ionones. Lactones present in propolis were generated by the esterification of corresponding hydroxycarboxylic acids and they are as followed: γ-nonalactone (coconut, creamy), γ-decalactone (coconut, creamy) and δ-octalactone (coconut, creamy). Terpenes including both mono- and sesquiterpenes are derived biosynthetically from isoprene. The ones present in propolis are as followed: linalool (floral), γ-terpinene (terpene-like), α-pinene (pine-like) and 1,8-cineole (eucalyptus). α- and β-ionones such as (E)- β-damascenone and β-ionone were generated by the oxidative cleavage of carotenoids.

8 Conclusion

Propolis samples (3 in total) collected over three consecutive seasons (Spring 2011, Spring 2012, and Spring 2013) were sensorially evaluated by trained panelists, using free-choice profiling and then a QDA. The aroma profiles were similar for each of the three years, all displaying typical propolis-like character. However, certain subtle differences were detected from the different samples. Propolis samples were individually ground into fine powders, extracted with diethyl ether, and subjected to SAFE distillation. After distillation, the aroma volatile isolates were concentrated to about 200 µL prior to performing cAEDA. When evaluated sensorially, the aroma isolates smelled similar to the authentic propolis samples and their sensory characteristics were found to be consistent with the results obtained for each year (1 through 3). Each propolis aroma isolate was subjected to a cAEDA, which resulted in a total of 47 aroma-active compounds in the FD factor range of 4 to 1024. Overall, the results of the cADEA and subsequent identification experiments resulted in a total of 13 odorants with relatively high FD factors (FD ≥ 256) in at least one sample. The highest FD factors were determined for 2-methoxy-4-vinylphenol (**41**) (clove-like), α-pinene (**3**) (pine-like), 1,8-cineole (**5**) (eucalyptus-like), (E)-2-nonenal (**11**) (green), (E,Z)-2,6-nonadienal (**14**) (cucumber-like), (E)-β-damascenone (**21**) (cooked apple), 2-methoxyphenol (**23**) (smoky), phenylethyl alcohol (**25**) (floral, rose), β-ionone (**26**) (floral, berry), eugenol (**39**) (clove-like), cinnamaldehyde (**31**) (cinnamon-like), (E)-ethyl cinnamate (**37**) (cinnamon-like), and 3-phenylpropanoic acid (**47**) (floral). Out of these odorants, 2-methoxy-4-vinylphenol (**41**) (clove-like) was determined to have the highest FD factor of 1024 for all three years. Although AEDA is an excellent method for discriminating the aroma-active compounds from the bulk of the

odorless volatiles, FD factors resulting from AEDA do not necessarily reflect the overall sensory importance of the compounds. As a result, further studies are needed to better approximate the overall sensory impact the aroma-active compounds identified in this present investigation. Quantitation of the key aroma active compounds by stable isotope dilution assay (SIDA), calculation of odor activity values (OAV, ratio of concentration to odor threshold), aroma reconstitution experiments and omission tests would be an excellent approach to gain more insight into the overall impact of individual odorants and groups of odorants present in propolis [148]. Of the total 47 odorants identified in the propolis samples, 39 were detected in all samples (years 1 through 3). Only 8 of the 47 total odorants were not detected in all 3 years. Although differences in FD factors were observed in propolis collected from each season, the overall temporal variation of odorants were remarkably consistent over a three year period (Table 7). To the best of our current knowledge, this investigation resulted in 22 compounds that are reported as aroma active compounds in propolis for the first time (Table 7).

9 Concluding Remarks

Taken altogether, the outcome of this work has provided considerable insight into the aroma active compounds that contribute to the odor of propolis (collected in central New Jersey) and on the influence of the seasonal changes on the composition of the aroma active compounds. Of significance, this work provides a valuable contribution on the

application of propolis to dietary and pharmaceutical industries in addition to the flavor and fragrance industries. The present investigation will also help to establish a basis for future research on the key aroma active compounds of propolis collected from different geographical locations and on the development of aroma standardization and quality control methods, which currently represent a major gap in the food, beverage, and consumer goods industry. Future investigations on the aroma of propolis from different geographic origins and from different bee species can utilize this present work as a starting point and a reference for aroma analysis.

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